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CYTOLOGICAL OBSERVATIONS ON
ENDAMOEBA BLATTAE

WITH EIGHT PLATES

BY
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CONTRIBUTIONS FROM THE ZOOLOGICAL LABORATORY OF THE
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I. INTRODUCTION

CELL STRUCTURE, and especially nuclear structure, is similar in the majority of metazoan groups. Detailed morphological differences are numerous, of course, even within comparatively restricted systematic limits, but these differences are confined to relatively unimportant minutae, and do not appear to be associated with far-reaching distinctions of taxonomic and phylogenetic importance. The basic morphological pattern of metazoan cells includes a vesicular nucleus with basophilic material suspended in a reticular or structureless matrix of karyolymph. Insofar as the writer is aware, the distribution of basophilic material coincides, or practically coincides, with the distribution of the material which reacts positively to the Feulgen nucleal test in all cases which have been studied. The majority of cytologists consider the basophilic nucleic acid-containing material as chromatin. During division almost all metazoan cells undergo mitotic phenomena which, but for slight variations in detail, follow the same course in all types of organisms. Meiosis, likewise, is similar in all Metazoa in its more general aspects. Thus we may consider the metazoan nucleus a comparatively fixed structural and physiological unit.

Such uniformity does not prevail among the Protozoa. Not only do distantly associated groups have nuclei with fundamentally different structure, as, for example, the vesicular nucleus of the Sarcodina and the massive macronucleus of the Ciliata, but relatively closely related forms, particularly among the Sarcodina, have nuclear patterns with significant differences. Although nuclear phenomena are but incompletely understood it appears that the divergence from the typical metazoan pattern, as observed in some groups, is quite fundamental.

Several factors appear to be important in bringing about nuclear diversity in Protozoa. One of the most important is the tendency toward nuclear specialization. In many of the protozoan groups nuclei are set aside for the consummation of specialized functions. Nearly all ciliates have macronuclei and micronuclei, apparently differentiated primarily for metabolic and reproductive functions respectively. A somewhat similar nuclear specialization is found in the trypanosomes, where a so-called kinetonucleus is distinguished from a trophonucleus, the former appearing to be associated with locomotive phenomena and the initiation of division, while the latter is associated with metabolic functions. Specialization of vegetative and generative nuclei is found among some of the Sporozoa. In the Myxosporidia nuclear specialization is carried still further. The developing pansporoblast contains shell nuclei and capsulogenous nuclei which appear to govern the differentiation of the spore membrane and polar capsule respectively. That so many cases

of nuclear specialization are associated with a segregation of reproductive from metabolic functions suggests that we have to deal with a segregation of reproductive chromatin or idiochromatin from somatic chromatin or trophochromatin. This concept was extremely popular at the turn of the present century and has since been utilized to interpret many nuclear phenomena among the various types of Protozoa. Since this possible separation of a "germ line" from a "somatic line" is not accompanied by histological differentiation as it is in the Metazoa, the nucleus itself must bear the brunt of the specialization.

Instead of specialization of whole nuclei the Amoebeae present a long series of slightly differing nuclear patterns which may be extremely simple, as in Vahlkampfia, or highly complicated, as in some of the Endamoebidae. This high degree of variability may be an expression of the tendency to segregate trophochromatin from idiochromatin. Morris (1936) suggests that this may be the case in *Endamoeba blattae*. Whatever the causes underlying the diversity of nuclear structure in the amoebae, the fact remains that nuclear morphology is an important taxonomic character. Relationships are postulated through the comparison of interphase and kinetophase nuclei. As might be anticipated the diversity of nuclear structure is accompanied by differences in the pattern which appears during division. A series extends from the simple, almost amitotic division of Vahlkampfia to highly complicated division types closely resembling metazoan mitosis, as in *Entamoeba histolytica*, and to equally complicated karyokinetic figures, as in *Endamoeba blattae*. So striking are the differences in these division figures that some investigators have hoped to learn something of the development of mitosis through the study of primitive types of amoebae. Most of the phylogenetic speculation dealing with the amoebae has been based primarily on a comparison of interphase and dividing nuclei.

As a result of the variation in the location and appearance of the nuclear elements of the amoebae, the distribution of the basophilic material, commonly known as chromatin, is extremely varied. The identification of chromatic elements in the nucleus is difficult in some cases, for the Feulgen nucleal preparation which is, to some extent, a test for thymonucleic acids, fails to react with the basophilic material found in the nucleus. Thus Morris (1936) and Sassuchin (1936) report that the nucleus of *E. blattae* was not colored by the Feulgen nucleal reaction, indicating that if any thymonucleic acids were present they were either chemically combined in such a way that the color reaction did not occur with the Feulgen reagents as applied, or that normal nucleic acids were present, but in quantities too minute to call forth a visible color reaction. Chalkley (1936) found that in *Amoeba proteus* the peripheral basophilic material was not colored by the Feulgen reaction, but that a cen-

trally placed karyosome reacted positively. The morphological relationship of chromatin—or at least the basophilic constituents of the nucleus—to nucleic acids is not understood in most members of the group.

Critical, detailed studies on the morphology of the interphase and dividing nuclei of different amoebae are essential if generic relationships and the implications of the differing structural patterns are to be understood. Such studies may reveal fundamental correlations between the distribution of basophilic constituents and of thymonucleic acids, and may shed some light on the functional significance they have in the consummation of nuclear activities during the life cycle of the organism.

It is with the problems outlined above that the present investigation deals. The large unusual nucleus of *E. blattae*, while attracting the attention of a number of investigators for the ease with which it can be studied, is still but incompletely known. A number of past investigations have resulted in contradictory results, and much work of confirmation alone is needed. The failure of the Feulgen nuclear reaction to reveal nucleic acid in the nucleus during the interphase is a striking example of the unusual conditions prevailing in this species. Sassuchin (1936) reported that he had failed to obtain a positive response to the Feulgen reaction, but suggested that studies of different stages in division and in the life cycle might reveal the presence of nucleic acid at particular stages.

Since the writer has used the Feulgen nuclear reaction a great deal in the present investigation, an explanation of his use and interpretation of this reaction may be valuable. It is an obvious criticism that this reaction is not a specific microchemical test of known validity for thymonucleic acids, as it can also react with other substances which are present in the cell. For example, plasmogen in the cytoplasm reacts positively. This substance was removed by exposing the slides to 95 per cent alcohol for from 12 to 24 hours. In some cases this substance was still present in small quantities, and the cytoplasm was colored lightly by the reaction. Since it occurs in the cytoplasm, however, it could scarcely be mistaken for nucleic acids occurring in the nucleus. A plasmogen reaction was observed in amoebae in which no reaction could be found in the nucleus, and in other amoebae in which no cytoplasmic reaction had occurred there was a nuclear reaction. Other cytoplasmic substances may react positively, but insofar as the writer is aware, the only substance which might react positively in the nucleus are the nucleic acids. Additional evidence in favor of the view that the reacting substance was nucleic acid was supplied by fixation in absolute alcohol and subsequent washing. According to Fischer (1899) and Mann (1902) nucleic acid is precipitated by alcohol. Slides of *E. blattae*, with control slides of the ciliates *Nyctotherus ovalis* and *Balantidium praenucleatum*, were fixed in absolute

alcohol and were found to react positively to the Feulgen test if there was a minimum exposure to water. The deep coloration of the macronuclear spherules of *Nyctotherus ovalis* was typical in all respects. These spherules are thought to contain nucleic acid (see Kudo, 1936). But nucleic acid precipitated by absolute alcohol is soluble in water, according to Mann and Fischer. Slides fixed in absolute alcohol and washed for 48 hours in running tap water failed to show a positive reaction for ciliates or amoebae in thin sections. Similar smear preparations were negative for the amoebae, and for the ciliates a faint coloration in the center of the macronucleus occasionally occurred in a few organisms lying in the thicker portions of the smear, while the majority failed to show any reaction. Just such a response to the technique used would be expected from nucleic acids. According to Mann and Fischer, nucleoproteins are also precipitated by absolute alcohol, but are insoluble in water after precipitation. This seems to offer some experimental proof in favor of the view that it was nucleic acid which reacted with the Feulgen reagents in the present study, and not nucleoprotein. With these few indications, and willing to admit that the specificity of the Feulgen reaction may be considered as doubtful, the writer considers the material in the nucleus of *E. blattae* which reacts positively to the Feulgen test to be, in all probability, nucleic acid. Until the development of new microchemical tests of greater validity for checking our results we are forced to work with whatever means are at hand. It is with this in mind that the present problem has been undertaken. The writer has interpreted the material reacting positively, then, throughout the present paper, as nucleic acid, not in the more strict chemical sense, but in a loose, cytological sense.

In this study a comparison of results proceeding from the use of basic dyes and the Feulgen reaction were made in order to determine the relationship of basophilic material to nucleic acids, as demonstrated by the Feulgen reaction, throughout the life cycle up to the formation of the mature cyst, following here the suggestion made by Sassuchin (1936) mentioned above, although when the work was undertaken the writer was unfamiliar with Sassuchin's publication. At the same time it was possible to search for new information concerning the morphology and life cycle of the amoeba, and to confirm some of the observations of earlier investigators. As a result of the study, in addition to details of nuclear division during the trophic and cystic stages, it has been possible to observe a cyclical variation in the quantity and distribution of nucleic acid, as determined by the Feulgen test, correlated with the processes of nuclear division and encystment. Several unusual relationships between the basophilic material and the nucleic acid-containing material have also been observed.

II. ACKNOWLEDGMENTS

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III. MATERIAL

Endamoeba blattae occurs in the anterior dilated portion of the colon of at least three species of cockroaches, *Blatta orientalis*, *Periplaneta americana*, and *P. australasiae*. The oriental cockroach was the source of the amoebae used for this investigation. It is very abundant on the University of Illinois campus and may be collected in large numbers from early spring to late fall. During the day the insects retreat into crevices in the walls of the buildings. At night they emerge to feed and can be captured easily with the aid of a flashlight.

They were kept in battery jars, from fifty to a hundred roaches living well in a single container. A variety of foods were tried, including Fleischman's yeast cakes, moistened soybean meal, potatoes, and apples. As reported by Kudo (1926) the yeast cakes form a most satisfactory diet, not because the insects fail to thrive on the other diets tried, but because the protozoan fauna respond unusually well to the yeast cake diet.

In cockroaches kept in a relatively crowded condition for several weeks in the laboratory the incidence of infection rose to almost 100 per cent. In nature, of course, such high incidence is not usually observed. Kudo (1926) reported that the percentage of infection is lowest in nature in spring and late fall when it falls to about 5 per cent. It is highest during the summer and early fall, reaching a peak of about 50 per cent in July, August, and September. Similar seasonal variation in incidence of infection of cockroaches just captured is recorded by the author. The incidence of infection was maintained at a high level throughout the winter in the animals kept in the laboratory, but for some reason it fell appreciably during early spring every year, although conditions under which the host animals were kept remained constant throughout. This was the more inexplicable since the large ciliate *Nyctotherus ovalis* was as numerous in these hosts as it had been during the

winter, and the smaller ciliate *Balantidium praenucleatum* appeared to be increasing in number at this time.

In immature hosts there is no appreciable difference in the incidence of infection between male and female insects. But a small percentage of adult males harbor amoebae, however, and a heavy infection in such hosts is extremely rare. The mature females, on the other hand, are as heavily infected as the immature insects and approach 100 per cent in incidence of infection. One factor concerned in this difference appears to be the small amount of food consumed by winged males, whose colons are frequently almost empty and greatly shrunken, in contrast to the large amount of food consumed by the voracious females, which have a very large and well filled intestinal tract.

IV. METHODS

LOCKE'S SOLUTION proved to be a very satisfactory medium for maintaining amoebae for periods long enough to complete the necessary procedures. The Locke solution was made with a reduced NaCl content (0.45 per cent) and other compounds reduced proportionately. This was the optimum concentration for survival as revealed by a series of tests for maximum survival time in varying dilutions. When observations were to be made *in vivo* the amoebae were kept in small (20 mm.) petri dishes in sterilized Locke plus 0.5 per cent to 1 per cent Difco albumin. They remained alive and active in this medium for several days if a part of the dissected colon was left in the culture. Many of the amoebae died after from two days to a week. The survivors appeared to adapt themselves to their environment and remained alive and more or less active after a month in many instances. Subculture was not effective, however, as multiplication was quite rare among the cultured amoebae.

Depression slide mounts were used only when a single organism was to be studied for an extensive period of time. Usually the amoebae were kept in petri dishes, removed for observation whenever necessary, and then replaced in the petri dishes. This method allowed greater freedom and proved to be less laborious than the depression slide method. It was especially well adapted to study with vital dyes, where it was not necessary to observe the same specimen continuously in some experiments. Vital dyes were kept in 1-1000 and 1-10000 solutions in Locke, and were diluted with Locke-Albumin to the desired concentration just before use. Of a number of vital dyes tried, Janus green B, Bismarck brown, brilliant cresyl blue and neutral red were the most satisfactory. The optimum strength for these dyes varied between 1-50000 and 1-100000. When a large amount of debris was present in the teased colon a larger amount of dye was needed.

TABLE I
SUMMARY OF NUCLEAR FIXATIVES USED

Simple (Uncombined) Fixatives		Compound Fixatives	
<i>Strong</i>		<i>Strong</i>	
1. Absolute Alcohol*		1. Gilson Carnoy*	
2. Glacial Acetic Acid*		2. Schaudinn*	
3. 10% Acetic Acid*		3. Carnoy*	
4. 40% Formaldehyde*		4. Gilson*	
5. Saturated Aqueous Mercuric Chloride*		5. Sublimate Alcohol*	
6. Saturated Aqueous Picric Acid*		6. Sublimate Acetic*	
7. 1% Chromic Acid*			
<i>Medium</i>		<i>Medium</i>	
8. 5% Acetic Acid*		7. Zenker*	
9. 2% Acetic Acid		8. Bouin*	
10. 3% Mercuric Chloride			
11. 1% Acetic Acid			
<i>Weak</i>		<i>Weak</i>	
12. 4% Formaldehyde		9. Flemming with Acetic	
13. 0.5% Acetic Acid		10. Flemming without Acetic	
14. 2% Osmic Acid		11. Champy	
15. Osmic Vapor		12. Altmann	
16. 70% Alcohol		13. Regaud	
17. 30% Alcohol			
18. Dioxane			

*Denotes that the fixatives were used at room temperature and at 45-50° C.
All others were used only at room temperature.

Permanent mounts for the study of cytoplasmic inclusions were made in toto and in sections 3 to 8 μ thick. Altmann, Regaud, Champy and Flemming without acetic were used as mitochondrial fixatives and were followed by Heidenhain's haematoxylin, Regaud's haematoxylin, Bensley's copper haematoxylin, Altmann's fuchsin-picric acid, Bensley's fuchsin-methyl green, Kull's fuchsin-aurantia-toluidine blue or Benda's alizarin-crystal violet. By far the most satisfactory stained mounts were obtained in material in which the whole colon was fixed with Altmann or Champy and sectioned at 3 to 5 μ , followed by staining in Benda's technique. Golgi material was studied with Kopsch and Kolatchev osmium methods and the da Fano silver technique.

Studies of the nucleus were made with living amoebae under oil immersion in bright and dark field illumination. Permanent mounts were made in toto and in sections cut from 2 to 10 μ in thickness. Except for the finer details of nuclear structure the thick sections proved more practicable.

A number of fixatives were used, chosen for the satisfactory quality of results obtained in preliminary trials. A summary of the fixatives used is given in Table I. The compound fixatives most used were numbers

1, 2, 3, 7, 8, and 9. A number of others not mentioned were tried and gave unsatisfactory results or were not used to any great extent because the results were not significantly different from those obtained with the standard protozoological fixatives.

Fixation and staining so completely alter the appearance and structure of the nucleus of *E. blattae* that it is impossible to describe the fixed and living nucleus in similar terms without very careful study. So many structures can be seen only in fixed nuclei that it was necessary to determine their position in the living nucleus before their interpretation was possible. The correlation between living and fixed nuclei was obtained by a study of the phenomena accompanying fixation. This study included all simple or uncombined fixatives shown in Table I, and numbers 1, 2, 3, 6, 7, 8, and 9 of the compound fixatives.

Amoebae were kept in Locke solution without albumin or were washed before use, for when fixative was added to a solution with albumin in it, the precipitation of the albumin obscured the amoebae. The organism selected was put on a slide in a small drop of Locke solution and a cover glass applied. By using the less viscous mineral oil in place of cedar oil it was possible to carry on observation under oil immersion. A piece of paper toweling was placed in contact with the cover slip at one side of the slide, to draw off the fluid until the amoeba was compressed, but still alive and able to move slightly. This compression was necessary, for amoebae not treated in this way were either carried away with the fixative, or were rendered too opaque for accurate observation. The nucleus was usually slightly compressed also, but insofar as could be determined the structure was not changed, and the parts retained their normal relationships.

The compressed nucleus was studied briefly under oil immersion. When the position of the various regions and structures had been determined, a drop of the fixing solution was placed in contact with the cover glass opposite the paper toweling, which drew it under the cover glass very rapidly. By this method the fixation could be watched under high magnifications and the results determined. Several times dark field illumination was used, but the precipitation of the protoplasm caused so much refraction that it was not continued as a routine practice. Indeed, even in the living nucleus nothing could be seen in dark field that could not also be seen in bright field illumination. When fixation was slow, a very rare condition, the sequence of the appearance of nuclear structures invisible in life was determined. Usually the process reached completion almost instantaneously, or at most in a few seconds, so that it was necessary to repeat many times, fixing the attention successively on

first one and then another region of the nucleus before the complete process could be ascertained. In order to identify the nuclear structures found, their affinity for basic dyes was determined at the end of the experiment by adding acetocarmine or methyl green to the preparation, after appropriate washing. During the whole procedure the nucleus was kept under constant observation.

This study of the process of fixation was very helpful in determining the relationship of the structures in the fixed nucleus to those observed in the living nucleus, and aided in obtaining a reasonably complete understanding of the morphological effects of fixation. By combining the study of the structure immediately following fixation with that of similarly fixed nuclei in stained permanent mounts, some idea of the effects of dehydration were also gained. Each fixative, simple or compound, so studied was used in preparing permanent mounts in toto and in sections, stained with Feulgen's nucleal reaction, Heidenhain's haematoxylin, and Safranin light green, following a rigidly standardized staining procedure.

A. FEULGEN'S NUCLEAL REACTION

After washing for 12 to 24 hours in 95 per cent alcohol the smears and sections were hydrated and passed into the hydrolizing solution where they were held for 5 minutes at 60° C., rinsed in cold hydrolizing solution 1 minute, put into the fuchsin sulphurous solution for one and a half hours, washed in 3 changes of sulphurous acid wash water, 10 minutes each, and in running dechlorinated tap water for 30 minutes. Following dehydration to 95 per cent alcohol the slides were lightly counterstained with light green (10 seconds in a slightly basic 0.5 per cent solution in 95 per cent alcohol) and mounted in balsam after clearing in xylol.

B. HEIDENHAIN'S HAEMATOXYLIN

Slides were mordanted for 5 minutes at 45° C., after which they were rinsed a total of 5 minutes in 3 changes of distilled water. They were stained in 0.5 per cent haematoxylin for 5 minutes at 45° C., washed in running dechlorinated tap water for 20 minutes, and differentiated in large lots in saturated aqueous picric acid. The destaining process could not be standardized perfectly because slight variations in thickness were sufficient to alter the time required to reach maximum contrast. A partial standardization was effected by differentiating large numbers of slides simultaneously, a very effective method for materials sectioned at the same thickness. The differentiation of smear preparations, naturally, could be but incompletely standardized even by this method.

C. SAFRANIN

After postosmication in used Flemming fixative for 15 minutes, and a wash of equal duration the slides were placed in Zwaardemaker's Safranin for one hour and washed in distilled water for 5 minutes. The preparations were then placed in 70 per cent alcohol for 30 seconds, 95 per cent alcohol plus $\frac{1}{2}$ per cent light green S. F. yellowish to deepest contrast (30 seconds to 1 minute according to fixation), rinsed by dipping into 95 per cent alcohol, left in absolute for 30 seconds, and passed into xylol.

Before the warmed haematoxylin method was settled upon for the standardized technique it was compared carefully with the cold method, using both mordant and stain for 12 hours. In all essentials the results were exactly comparable, differing only in the tint of the result. The warmed haematoxylin tended to give deep violet-black nuclear structures, while the cold method produced an even deeper brownish black. Similarly various agents for differentiating haematoxylin were tried. Picric acid gave the sharpest differentiation, and iron alum was next best.

For purposes of comparison a number of other staining techniques were tried. Paralleling the standardized techniques used with the simple fixatives a series of slides stained with Giemsa were made. Occasionally other stains were tried with the simple fixatives, but none were used consistently enough to allow any interpretation of results. Insofar as could be seen with the incomplete data from the other stains, no further structure could have been identified with the other stains tried. With the compound fixatives, however, many combinations were used. These are summarized in Table II.

The large number of stains and fixatives used made it possible to check the results of nearly all the earlier investigators with preparations treated with the same reagents they had used, or with comparable techniques, as determined by a comparison of the effects of different fixatives and stains. This has led to the explanation of some of the discrepancies found in their work.

It became apparent from the study of the material that the nuclear morphology was sometimes altered strikingly by the fixative and stain used. The important differences observed in nuclear appearance after different commonly used fixatives had been employed led to the study of the effects of the simple fixatives, in the hope that some explanation for these discrepancies might be gained. In many cases the action of compound fixatives could be explained from the study of the reagents composing them. The simple fixatives tried are summarized in Table I.

Each simple fixative was studied in the same way. The immediate effects of fixation were determined by observation of the process of fix-

TABLE II
SUMMARY OF STAIN-FIXATIVE TECHNIQUES USED

<i>Nuclear Studies</i>	
Stain	Fixatives*
1. Delafield's haematoxylin.....	1, 2, 3, 4, 5, 6, 8, 9.
2. Ehrlich's haematoxylin.....	1, 2, 3, 4.
3. Heidenhain's haematoxylin.....	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11.
4. Borax Carmine.....	1, 2, 3, 4.
5. Paracarmine.....	1, 2, 3, 4.
6. Giemsa.....	1, 2, 3, 4, 5, 6, 7, 8, 9.
7. Safranin.....	3, 5, 6, 8, 9, 11.
8. Safranin-light green.....	3, 5, 6, 7, 8, 9, 10, 11.
9. Safranin-orange G.....	9, 11.
10. Flemming's Triple.....	3, 6, 8, 9, 11.
11. Feulgen's nucleal reaction.....	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11.
<i>Cytoplasmic Studies</i>	
12. Heidenhain's haematoxylin.....	10, 11, 12, 13.
13. Regaud's haematoxylin.....	11, 12, 13.
14. Bensley's copper haematoxylin.....	11.
15. Altmann's Fuchsin-Picric Acid.....	10, 11, 12, 13.
16. Bensley's Fuchsin-Methyl Green.....	10, 11, 12, 13.
17. Kull's Fuchsin-Aurantia-Toluidin Blue..	10, 11, 12, 13.
18. Benda's Alizarin-Crystal Violet.....	10, 11, 12, 13.

*The numbers refer to the numbers found in Table I in the Compound Fixative column.

ation under oil immersion, using the technique described above for compound fixatives. Smear preparations fixed with the simple fixatives were made and stained with the three standardized procedures outlined above. Sections $10\ \mu$ thick were also stained with these procedures. These permanent mounts were divided into two lots. The first lot was made with a view toward exposing the preparations to a minimum contact with water, at least before the staining process was inaugurated. The second lot was washed for prolonged periods of time in running dechlorinated tap water before staining. Smears were all washed for 24 hours. At first an attempt was made to wash sections for 24 hours also, but later the whole colons were washed for two days before sectioning, and the sections were washed for an additional 12 hours just before they were stained.

As had been expected from the available knowledge concerning the effects of fixation on certain of the more common chemical substances occurring in the nucleus, some of the structures precipitated by simple fixatives were soluble in water, while others were not. By comparing the results obtained from the study of washed and unwashed preparations with the tables of soluble and insoluble substances precipitated by the various fixatives in Mann (1902) and Fischer (1899), it was possible to come to some preliminary and tentative conclusions concerning the com-

position of certain of the nuclear elements. These results were also used to explain the effects of some of the compound fixatives.

V. GENERAL MORPHOLOGY

Endamoeba blattae is one of the largest species of endozoic amoebae known. It attains a maximum size of over $200\ \mu$ and a minimum size of about $45\ \mu$ in the trophic stage. Size distribution varies markedly between these extremes and is affected significantly by the diet of the host. Starvation of the host reduces the number and size of the amoebae. An apple diet also causes a reduction of size, although to a lesser degree. In either case there is a distinct reduction in the number of food inclusions, resulting in beautifully transparent forms. Under such conditions the mean size distribution may shift from 10 to $15\ \mu$ or more. When precystic and cystic amoebae are not present the number of smaller trophic forms is usually distinctly reduced. The highest size frequency usually occurs between 80 and $100\ \mu$ in well fed hosts.

Before the work of Lucas (1927) all amoebae in the cockroach colon were considered as belonging to a single species, *E. blattae*. Thus the size range of this species had always been extended to include the much smaller forms, *Endamoeba thomsoni* and *Endolimax blattae*. Grassi (1882), for example, mentions a number of small amoebae 4.4 to $6.6\ \mu$ in diameter. From his brief description of these tiny organisms it is apparent that he must have observed *Endolimax blattae*. Other early investigators record similar minute dimensions. Some of these investigators believed that the tiny amoebae were recently excysted *Endamoeba blattae*, a view which may have had some basis in fact according to the results of Morris (1936), who describes extremely small, transparent amoebae which emerge from the cyst. Morris was the first to record the size range of the trophic amoebae which has been observed in this study, setting the lower limit of size for the trophic forms at about $50\ \mu$. Precystic amoebae are much smaller than trophic forms, ranging from about 20 to $50\ \mu$, with the largest number between 30 and $40\ \mu$ in diameter. The cysts measure from 15 to $35\ \mu$ in diameter, with the mean around $25\ \mu$.

Cytoplasmic differentiation is apparently of two kinds. A clear, hyaline ectoplasm contrasts with a more granular vacuole-filled endoplasm. A plasmasol-plasmagel differentiation may also be observed. In addition to these, Schubotz (1905) described a so-called "light" and "dark" plasma differentiation. From his descriptions and figures it appears that the dark plasma was the plasmagel and the light plasma the plasmasol, for the distribution of the two types of plasma appears to be identical, as Morris (1936) was led to conclude. Bütschli (1878) and Schubotz (1905) were

unable to distinguish a distinct ectoplasmic layer. Most of the other investigators have observed an ectoplasmic differentiation, however, and Kudo (1926) discusses this point thoroughly. As he points out, a distinct ectoplasmic sheet is but rarely visible and occurs only in comparatively sluggish or inactive amoebae. Small regions of ectoplasm may occur in the anterior or anterolateral margins of active amoebae, but a distinct ectoplasmic layer is not found.

Under high magnifications the plasmasol-plasmagel differentiation is distinctly visible. The plasmagel appears as a more or less continuous reticulum throughout the amoeba in active forms. It is somewhat lighter in color than the plasmasol in life, and stains more intensely with acid dyes, as can be seen in sectioned preparations (Fig. 65). Within the interstices of the plasmagel reticulum lies the plasmasol, a rather granular substance in life, which stains less intensely with acid dyes. Not infrequently a posterior region containing much plasmagel and very little plasmasol can be distinguished in actively motile forms.

During active forward movement striae frequently appear in the cytoplasm. They appear only in active amoebae, and run parallel to the direction of flow of the cytoplasmic currents. These have been described by almost every investigator, most of whom have offered some explanation for their occurrence. Bütschli (1878) and Schubotz (1905) believed that the striae are fibrils formed by longitudinally oriented alveolar walls. Kudo (1926) postulated a stretching of the alveoli in the direction of flow caused by the active endoplasmic streaming which resulted in the striated appearance. Morris (1936) suggested that the plasmasol and plasmagel are immiscible and that the striae are produced by streamers of plasmagel extending into the plasmasol. No observations have been made which will support any of these contentions directly. Concerning the last mentioned possibility, however, it may be noted that streamers of plasmagel do sometimes reach far forward from the posterior gelled region of the amoeba. These have been observed many times in actively moving amoebae, even when striae, which can be seen under quite low magnifications, unlike the plasmagel streamers, could not be found. In other cases striae have been observed with the streamers of plasmagel extending forward between them. The writer is, therefore, of the opinion that the very conspicuous striae are distinct from the delicate streamers of plasmagel. Bütschli (1878) noted that the striae tend to disappear when the amoeba is slightly compressed, which has been amply confirmed during the present study. This observation seems to be inexplicable on the basis of any theory advanced so far, and until further information is available the problem of the nature and mode of formation of the striae must be considered as unsolved.

Locomotion and endoplasmic streaming are very striking in *E. blattae*,

and have been the subject of considerable study. As early as 1898 Rhumbler studied some of the phenomena associated with pseudopodial formation in *E. blattae*. He described cytoplasmic currents accompanying locomotion as "fountain-streaming," characterized by a strong backward flow of plasmasol beneath the relatively thin outer shell of plasmagel. Pseudopodial formation, he noted, is often almost explosive, the current being very rapid and strong.

The speed of forward progression may be quite rapid. Kudo (1926) noted an amoeba which traversed twice its body length in one minute, and several which travelled distances well over one body length per minute. Similar speeds were observed during the course of this study. It appears that active amoebae usually average about one body length per minute, unless very large or hampered by much debris. During active movement the shape assumed is similar to that of *Vahlkampfia limax*. There is a single broad pseudopod, the advancing edge of which may be tipped by a narrow band of ectoplasm or have small anterolateral margins of ectoplasm. Occasionally two pseudopods are formed. Among active amoebae this indicates a sudden change in the direction of movement. Large numbers of lobose pseudopodia, as are formed by *Amoeba proteus*, have not been observed.

In actively moving individuals endoplasmic streaming involves, as explained by Rhumbler, axial and superficial cytoplasmic currents. The axial current advances, moving from the posterior region of plasmagel toward the anterior margin of the pseudopod. There it breaks, fountain-like, to form superficial currents which run posteriorly just under the thin outer layer of plasmagel. A very little of the protoplasm streaming backwards may sometimes return directly into the axial stream, forming an eddy. These eddies, which are comparatively rare, are very striking in amoebae stained with neutral red. The great majority of the plasmasol flowing toward the posterior end of the body is converted into plasmagel at the middle of the body or somewhat anterior to it. Morris (1936, p. 231) says, "Although forward movement of the animal masks the fact during locomotion, it can clearly be seen, when the amoeba is attached by the uroid, that the material which streams forward through the center turns at the anterior end and streams backward along the periphery. There is apparently no anterolateral gelation, nor corresponding reliquefaction in *E. blattae*." The writer cannot confirm this point for amoebae moving actively in a relatively clear field, although the opinion was maintained during the early part of the work. During the observations made on vitally stained amoebae it was possible to trace the course of single granules in the endoplasm during their circuit through the cytoplasm. Granules always showed approximately the

same course. They flowed forward from the posterior end until they reached the anterior tip, where they turned and started to flow backward. The backward flow continued from one-third to one-half of the length of the amoeba during very active movement, and somewhat less during slower movement. At this time the granules ceased to move backward. Using slides with scratches on the surface it was possible to demonstrate that the granules did not change in relation to the substratum from this time until they began to flow forward again, which seems to indicate that a lateral gelation had taken place. It appeared to the writer that at the point at which the supposed gelation occurred there was a reduced amount of Brownian movement. The only exceptions to this were the few granules which became caught in the cytoplasmic eddies which occasionally are found at the anterior end. These enter almost immediately into the anteriorly directed current without continuing backward to the region of gelation. After this had been observed it was sought for in amoebae which were not vitally stained, lest the gelation was an effect of the staining process. With some difficulty the observations were tentatively confirmed in unstained amoebae, although the difficulties in observing unstained small granules made it impossible to be entirely certain of the results.

Not all amoebae show currents which are exactly like those just described. If an amoeba is caught in a mass of detritus, or if it is attached to the slide at one end by the "uroid," streaming without gelation occurs. This confirms the description given by Morris (1936), cited above. It is not a normal locomotory current, however, in that it does not lead to forward progression. When organisms undergo this peculiar type of streaming it sometimes occurs that the superficial backward current flows along only one side of the organism. This results in displacing the usually central axial stream toward the opposite side, and gives the amoeba a peculiar spiral appearance. As when attached by the uroid, this type of streaming apparently does not lead to forward progression. It appears to be identical with the so-called "spiral streaming" described by Sassuchin (1930). It has been observed that during "spiral streaming" gelation in the lateroposterior region of the amoeba occasionally occurs, although it is usually repressed.

Rhumbler's explosive pseudopodial formation in relatively inactive amoebae has been observed frequently. It involves a rapidly moving current of endoplasm breaking through the comparatively rigid ectoplasmic sheet. It is interesting to compare this phenomenon with the formation of pseudopodia in *Amoeba proteus* as described by Mast (1926). He describes a hyaline cap, which is formed as the endoplasm breaks through to the ectoplasmic layer. This seems to be directly analogous to the mode

of formation of the "explosive pseudopodia" in *E. blattae*. It is even more striking in the latter, however, because of the stronger endoplasmic currents and the heavier layer of ectoplasm.

Inactive amoebae are frequently rounded, and bear a large number of small papilliform ectoplasmic protuberances which appear to be formed by the explosive process. In these cases the plasmasol which burst through the gelated region has become gelated and shows no further streaming. Not infrequently one or more of these may be drawn out into a very long pseudopodium containing only hyaline ectoplasm. These are formed very slowly and appear to have no association with locomotion, for the organisms lie quiescent during the whole process. Actively moving amoebae never possess these long hyaline pseudopodia, which persist for but a short time after the organisms bearing one or more of them begin to undergo progressive locomotion. These long pseudopodia appear to be identical in structure with the small ones. Kudo (1926) gives a very accurate description of the small pseudopodia.

VI. CYTOPLASMIC INCLUSIONS

THE PROTOZOAN cytologist has much difficulty in understanding and interpreting cytoplasmic inclusions. So much cytoplasmic differentiation has occurred that many structures not homologous to any found in metazoan cells occur. Some of these structures show affinity for osmium and silver, which is the most important test for Golgi material in metazoan cells. In some cases, as the silver line system of ciliates, no difficulty is met with in distinguishing cytoplasmic inclusions from the unusual specializations of the protozoans. In other cases, particularly in some of the kinetic elements of the flagellates, there has been no unanimity in interpretation, and many discrepancies appear in the literature. Where the cytoplasmic inclusions resemble those found in the metazoan cells which have been studied, interpretation is not so difficult. The osmiophilic material in the cytoplasm of the Sporozoa does resemble osmiophilic material found in invertebrate cells, and it is not difficult to suppose that the two are homologous. But the osmiophilic material around the contractile vacuole of some ciliates is quite different in all respects. Nevertheless it is interpreted by some investigators as being homologous with metazoan Golgi. An added complication has developed from the study of the neutral red vacuome, declared by some cytologists to be the Golgi material and by others to be distinct from it. As a result of these various difficulties there is much confusion prevailing among protozoologists with respect to the Golgi material.

In two groups of Protozoa, the Sporozoa and Sarcodina, the identification of Golgi material seems to be less troublesome than in the other

groups. The morphological similarity of the osmiophilic and the argento-philic inclusions to those found in the metazoan cells aid a great deal in interpreting results. A further aid is found in the fact that there is almost a complete absence of other structures which resemble the Golgi material only in their ability to reduce osmium or silver.

In spite of, or perhaps because of, the relative ease with which Golgi material can be studied in these two groups, it has been rather neglected. A considerable amount of work has been completed on the Sporozoa, but studies on Sarcodina are very few. Causey (1925) studied *Entamoeba gingivalis*. Brown (1930) studied *Amoeba proteus*. Hirschler (1927) made observations on *Endamoeba blattae*. A few added observations have been made by Mast and Doyle (1935), on *Amoeba proteus*. Otherwise the amoebae are wholly unknown. In view of our incomplete knowledge of the Golgi material in the amoebae, *E. blattae* has been restudied, using osmium and silver techniques, with an attempt to correlate the results with those obtained with neutral red and other vital dyes. Some observations were also made on the mitochondria.

A. MITOCHONDRIA

In amoebae stained vitally with Janus green B the mitochondria are stained a light green. They appear as slender rods, usually rather short, which flow about freely in the cytoplasm during the streaming which accompanies movement. The mitochondria appear to be rather adhesive. They are often found adhering to other cytoplasmic granules, and in amoebae stained with Janus green B and neutral red the granules stained red often adhered to the mitochondria. In view of these observations the fact that the mitochondria are occasionally found adhering to the wall of the food vacuoles does not appear to be significant. In many cases there were no mitochondria found on small vacuoles, and many of the mitochondria were not associated with vacuoles. It appears to be safe to conclude that no direct relationship between mitochondria and food vacuoles has been observed for *E. blattae*.

After fixation the mitochondria are shorter and heavier. They usually lie in a small clear area in the cytoplasm. This may be a shrinkage phenomenon. Although no dividing mitochondria were found in vitally stained material, in permanent slides evidences of division have been found. Dumbbell-shaped mitochondria have been found; in several instances two small mitochondria were found in a single clear area. This seems to indicate that the mitochondria divide. No relationship whatever could be found between the occurrence of division in the mitochondria and the stage of nuclear division. During the early part of the precystic period the mitochondria appear to be as numerous as, and morphologically

identical with, the inclusions found in the trophic amoebae. Later precystic and cystic stages have not been observed. Early observations seem to indicate a diminution in number and size of mitochondria in the mature cysts.

B. OSMIOPHILIC AND ARGENTOPHILIC INCLUSIONS

Studies on the osmiophilic inclusions of *E. blattae* were first made by Hirschler (1927). He found two types of osmiophilic inclusions. The larger inclusions were composed of two types of material. An outer osmiophilic region, circular or crescentic in optical section, was contrasted with a more or less spherical inner region of osmiophobic material in the larger type of inclusion. These inclusions measured about $3\ \mu$ in diameter. In addition to the larger inclusions there were a number of smaller inclusions which were quite variable in shape and were restricted to the plasmasol or the dark plasma of Schubotz.

Granules and larger inclusions, apparently identical with those described by Hirschler, were found. His descriptions and figures leave no doubt that the inclusions found were the same as those he had observed. According to Hirschler the smaller granules were lighter than the osmiophilic shell of the larger inclusions. In the material prepared for this study the two osmiophilic components—the smaller inclusions and the osmiophilic shell of the larger inclusions—were approximately equally dark. The difference noted here may be explained by the fact that Hirschler appears to have differentiated further than was the practice in this study.

Argentophilic material appears to be identical in all respects with the osmiophilic material. No difference in appearance or distribution could be found.

A comparison of the osmiophilic material found in *E. gingivalis* as described by Causey (1925) with those found in *E. blattae* shows rather distinctive differences. Causey found a large reticulum which apparently originated from smaller crescentic bodies. These smaller crescentic bodies seem to be similar to the larger inclusions found in *E. blattae*. Small granules which Causey found associated with the food vacuole led him to conclude that these small granules are the primordia of the Golgi material, which is formed from them. Causey's work has been questioned by several investigators, however, who feel that his methods and techniques were not standardized. Thus Nigrelli and Hall (1930, p. 21) say: "The technique used by Causey was not one of the methods used commonly for demonstration of Golgi material and Bowen (1928) has pointed out that Causey's method ordinarily would not be expected to demonstrate elements of the Golgi apparatus. Thence, the occurrence of a

filamentous or net-like 'Golgi apparatus' in *Entamoeba gingivalis* cannot be accepted definitely until Causey's observations have been checked by some of the more commonly used methods for demonstration of Golgi material." Nigrelli and Hall have studied Arcella, and found in that member of the Sarcodina a group of bodies not unlike the larger structures found in *E. blattae*. Brown (1930) found a number of osmiophilic and argentophilic granules accompanied by a smaller number of larger bodies composed of an osmiophilic cortex and an osmiophobic core in *Amoeba proteus*. The larger bodies described by Brown appear to be morphologically similar to those found in *E. blattae*, and the smaller granules also seem similar to the smaller inclusions found in this species.

Exclusive of the work of Causey, then, Golgi material, or, at least, osmiophilic material appears to be generally distributed in two forms. Smaller granules of osmiophilic substances are dispersed in the cytoplasm in large numbers, and larger structures consisting of osmiophilic and osmiophobic parts occur in smaller numbers. The smaller granules may be lacking in Arcella, as Nigrelli and Hall do not mention them. The exceptional case of *E. gingivalis* requires further study before any interpretation can be made. It is interesting to note that the osmiophilic material in the Sporozoa which have been studied is likewise composed of two types of inclusions similar to those found in the Sarcodina (see Hirschler, 1927 and Bowen, 1928).

C. NEUTRAL RED-STAINABLE INCLUSIONS

The use of neutral red as a vital dye for the demonstration of Golgi material has met with some opposition. Neutral red was originally thought to demonstrate the vacuome, supposedly distinct from the Golgi material. The fact that the vacuome, which stains with neutral red, is able to bring about a reduction of osmium has led to the formulation of the opinion that Golgi and the vacuome are either closely related or identical. Hall and his students have found that the neutral red-staining inclusions are identical with the osmiophilic material in a number of protozoan species, including Arcella.

In the case of *E. blattae* no report has been found in the literature concerning the results of intra-vitum staining with neutral red. In dilutions of 1-50000 to 1-100000 it was found that a number of small granules were specifically stained. These small granules were floating freely in the endoplasm. In addition to the granules a number of larger spherical bodies measuring from 3 to 3.5 μ in diameter were stained, although less intensely than the smaller granules. The granules usually acquire a red-violet color in fifteen to twenty minutes exposure, while

after this period the spherical bodies are an orange red. This intensity of color may represent a greater dilution of the stain in the larger bodies, or may possibly indicate a different pH in the two types of inclusions. Almost every one of the larger spherical inclusions have from one to five or six of the smaller granules attached to the periphery. A spherule without at least one granule has never been found. The difference in staining intensity makes it very easy to determine whether or not there are granules adhering to the surface of the spherical bodies.

The spherical bodies are not stained permanently by the neutral red. Their color reaches its greatest intensity in from 15 to 30 minutes after the dye is first applied to the amoeba, when they are a deep orange red shade. This typical coloration persists for several hours, and then gradually becomes less intense. In from 6 to 24 hours colorless spherical inclusions may be found with several intensely stained granules adhering to them. It is possible that this is due to the oxidation of the stain to a colorless leucobase. The smaller granules retain their deep red-violet coloration until after the death of the amoeba.

In the precystic amoebae the conditions are quite different from those just described. The smaller granular inclusions are present in great numbers, distributed at random throughout the cytoplasm. The larger spherical inclusions, however, are not to be found, at least after the nuclear transformation which accompanies the precystic development is completed. During the study of the vitally stained amoebae it was believed that some connection might exist between the larger spherical inclusions and the food vacuoles. This was especially noticeable in several well-fed amoebae from potato-fed hosts. In these specimens a large number of the spherical inclusions were found adhering to the walls of the food vacuoles containing grains of potato starch. The complete relationship of vacuole and inclusion is not as yet understood. It is interesting in this connection to observe that during the precystic period nearly all and finally all of the food vacuoles disappear at the same time that the spherical inclusions disappear. This appears to strengthen somewhat the inference that there is some relationship between the vacuoles and the inclusions.

Granules and spherules, apparently identical with those described above, were also stained with brilliant cresyl blue and Bismarck brown. These two dyes were so much less specific than neutral red, and the amoebae die so much more rapidly that few observations were made with them. No decolorization of the spherical inclusions was observed in amoebae stained with these dyes, but whether this depended on the greater toxicity of the stains or a difference in the chemical nature of the dye could not be determined. A number of other stains were tried.

Unfortunately they were either ineffective or stained so many structures that it could not be determined whether they stained the neutral red-stainable inclusions.

D. INFERENCES

The similarity in size, appearance, and general distribution of the inclusions staining with neutral red and those which were impregnated with osmium and silver appears to be in accord with the observations of Hall and his students on various species of Protozoa. Insofar as this writer is aware, this information was previously available for but one member of the Sarcodina, namely *Arcella*. A number of times confirmation was obtained by impregnation of vitally stained amoebae with osmium. In all cases the stained inclusions were darkened with reduced osmium. Insofar as could be determined such preparations did not differ from the usual Golgi slides in general appearance and distribution of the impregnated inclusions.

The fact that there were two distinct types of inclusions, both of which were stained by neutral red and impregnated with osmium and silver, made it extremely difficult to interpret the results. It has been felt that until some additional information is available regarding the osmiophilic inclusions it would be impossible to determine accurately which type of inclusion is to be considered as a homologue of the metazoan Golgi material. Certainly not all osmiophilic inclusions are Golgi material, and supplementary work must be carried out before any conclusions can be reached.

It is extremely interesting, however, to observe a similarity between the larger type of osmiophilic inclusion and the secretory granules found in some metazoan gland cells. Sharp, in his *Introduction to Cytology* (1934), shows figures of secretory granules from various sources (Fig. 37, p. 76) which appear to be identical insofar as structure is concerned with the larger inclusions occurring in *E. blattae*, *Amoeba proteus*, and *Arcella*. Some of the Sporozoa, also, have osmiophilic inclusions which are similar to the secretory granules. The question as to whether this is merely a coincidental resemblance, or is significant, cannot be settled on the basis of the present data. If, as it appears possible, some relationship between the food vacuole and osmiophilic inclusion can be found, it would seem that there would be an even greater resemblance of the secretory granule to the larger osmiophilic inclusion.

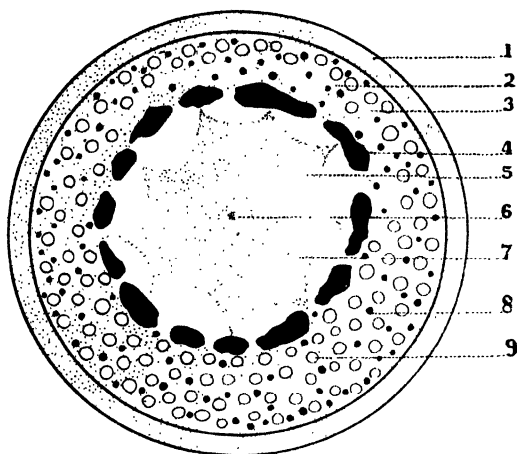
The smaller type of osmiophilic inclusion of *E. blattae* appears to cling to the outside of the larger spherical bodies in amoebae stained with neutral red. The writer believes it possible, at least, that the osmiophilic crescent of the larger spherules may be no more than the accumulation and imperfect preservation of the small granules upon the larger inclu-

sion. This, again, appears to afford another resemblance of the larger inclusion to secretory granules, for it has been supposed that the osmiophilic portion of the secretory granules represents a portion of the Golgi network which remained in contact with the secretory granule after it had been formed. If this relationship of osmiophilic granule to the larger spherical inclusion should prove, on further work, to be correct, it appears that the small granules would be the true Golgi homologue. It is, of course, impossible to conclude, on the basis of the evidence at hand, that the larger inclusions are secretory granules or Golgi material, or that the smaller inclusions are the Golgi homologue, and we await further investigations of the functional relationships of the granules to the spherical inclusions and of the spherical inclusions to the food vacuoles which may possibly throw some light on the question.

VII. TERMINOLOGY USED FOR NUCLEAR DESCRIPTION

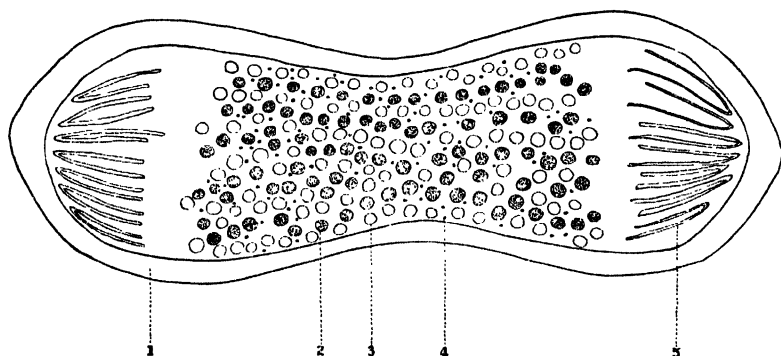
IN ORDER to facilitate interpretation of the descriptive text a diagrammatic representation of the trophic interphase and kinetophase nuclei are given in Text Figs. I and II, with the structures fully labelled. The terms selected, because of the structural differences between the nucleus of *E. blattae* and that of other parasitic amoebae, are in some cases inapplicable if strict homology of similarly designated structures is expected. To preserve the customary terminology as much as possible some concepts have been rather strained. Indeed, in many of the nuclear structures of the Sarcodina great confusion prevails insofar as homology is concerned, and few investigators have attempted to erect a consistent terminology. In the case of *E. blattae*, the previous investigators have frequently referred to the same structures with very diverse terms. Whenever feasible the terms employed by the majority of previous workers are used. Definitions and remarks concerning the terms selected are given below to facilitate comparisons with the descriptions of previous investigators.

Central ground-plasm.—This substance fills the inner part of the intranuclear space. Although not technically a ground-plasm, since the chromosomes are formed from it, its clear, transparent optical character in life and its homogeneous or delicately reticulated, undifferentiated appearance in the fixed condition during interphase give the term some descriptive value. It forms the homogeneous, granular or reticular region which lies within the endosomal girdle in fixed material. It is optically differentiated from the peripheral ground-plasm, with which it is connected at a few points by radii-like extensions in fixed material, or is in contact along the whole margin in material fixed with weak fixatives or in life (Text Fig. I).



TEXT FIG. I. SCHEMATIZED DIAGRAM OF NUCLEUS OF TROPHIC *E. blattae*

(1) Nuclear membrane; (2) peripheral zone; (3) peripheral ground-plasm; (4) endosome; (5) central region; (6) centriole; (7) central ground-plasm; (8) peripheral granule; (9) peripheral spherule.



TEXT FIG. II. SCHEMATIZED DRAWING OF DIVIDING NUCLEUS OF TROPHIC *E. blattae*

(1) Nuclear membrane; (2) endosomal spherule; (3) peripheral spherule; (4) peripheral granule; (5) chromosome.

Central region.—The region lying within the endosomal girdle in the interphase nucleus. This is identical with the central zone of Sassuchin (1936) (Text Fig. I).

Centriole.—The small basophilic granules which may sometimes be seen at the center of the central ground-plasm in the interphase nucleus and at the poles in the kinetophase nucleus. Although it could not be

traced accurately throughout karyokinesis, its position and appearance invariably resembled that of a centriole. This is identical with the central granule of Kudo (1926) and the karyosome of Morris (1936) (Text Figs. I and II).

Chromosome.—The strands which appear at the poles of dividing nuclei, and whose anlage may be seen in the central region during early division stages. They contain free thymo-nucleic acid as determined by the Feulgen nucleal reaction, but are not intensely basophilic. Their failure to stain deeply with basic dyes and the fact that they never pass through a metaphase plate stage shows that they are not entirely homologous in their reactions to the metazoan chromosomes, but since they appear to be functionally identical they merit the term chromosome. Morris (1936) and Janicki (1909) also use the term chromosome for these structures (Text Fig. II).

Chromosomal strands.—Slender strands of discrete granules which appear in the central ground-plasm during early division stages. They appear to be the anlage of the chromosomes (Fig. 25).

Endosomes.—Large basophilic bodies lying between the peripheral and central regions. The question of their relation to nucleolar substance is discussed elsewhere. They have been termed pseudochromosomes (Morris, 1936, and Mercier (?) 1910). Kirby (1927) terms similar structures in amoebae from termites nucleoli (Text Fig. I).

Endosomal anlage.—The elongate heterophilic strands from which the endosomes develop during early interphase. They appear during very late telophase (Figs. 1, 2, 3; see p. 132).

Endosomal spherules.—The small spherules of basophilic material found in dividing nuclei. They occupy the median part of the constricting nuclei and are frequently arranged in irregular rows (Text Fig. II).

Hyaline body.—The mass of hyaline material lying at the poles of late kinetophase and early interphase nuclei, associated with the dedifferentiating chromosomes. This structure seems to be distinctive, no such nuclear element being described in other parasitic amoebae. It appears to be identical with the karyosome of Janicki (1909) (Figs. 9, 10).

Interphase.—The metabolic period during which no reaction with the Feulgen reagents can be demonstrated, indicating that there is no free thymo-nucleic acid, or that it is present in but very small amounts.

Kinetophase.—The division period. Lack of a metaphase makes it inadvisable to attempt to differentiate prophase and anaphase, so the whole division period is considered together under the term kinetophase. Early kinetophase signifies the period before the typical concentric arrangement of the nuclear elements is disrupted. Late kinetophase signifies the

period following the clumping of the chromosomes at the poles of the nucleus. Middle kinetophase refers to the period between these two stages. The kinetophase is considered as completed when the reaction to the Feulgen reagents is no longer demonstrable.

Peripheral granules.—The basophilic granules lying in the peripheral ground-plasm between the nuclear membrane and the endosomes (Text Fig. I).

Peripheral ground-plasm.—The ground substance between the endosomes and the nuclear membrane, containing the peripheral granules and peripheral spherules (Text Fig. I).

Peripheral spherules.—The refractive spheres which lie in the peripheral ground-plasm in living nuclei. They are resistant to staining with acid or basic dyes (Text Figs. I and II).

Peripheral zone.—The region between the nuclear membrane and the endosomes (Text Fig. I).

VIII. THE TROPHIC NUCLEUS; HISTORICAL REVIEW

A. INTERPHASE

Nuclear membrane.—The unusually heavy nuclear membrane is reported by all investigators as measuring from 1 to 2 μ in thickness. A refractive, homogeneous structure is described by these investigators for both living and fixed nuclear membranes. Bütschli (1878), who gave the first description of *E. blattae* from living material, described a delicate membrane which invested the nuclear membrane, separating it from the cytoplasm. This has never been confirmed. According to Schubotz (1905) the nuclear membrane appears to be bilamellar in some cases. He observed a dead organism in which the membrane had separated into a thin inner and heavier outer layer. This is unconfirmed by later workers. A distinct beak-like projection has been seen protruding from the nucleus by all the previous investigators. Janicki (1909) observed that the membrane is thinner at that point. Although no one has since definitely confirmed this, the illustrations in several publications by others have shown clearly that the projection has a thinner membrane than the remainder of the nucleus. No further contributions to the morphology of the nuclear membrane appeared until 1930, when Sassuchin reported that it had a striated structure in fixed and stained preparations. He believed that he could demonstrate a comparable physical structure in living nuclei studied with dark field illumination. These striations, he suggested, may represent a system of canals connecting the cytoplasm and the nucleoplasm. No investigator has confirmed his observations.

Peripheral zone.—Bütschli described the peripheral zone as a finely granular-reticular region. Schubotz (1905), who was the first to give a detailed account of the morphology of the nucleus in fixed and stained condition, made a careful search for nuclear structures and attempted to determine the nature of the elements he saw with artificial digestion experiments. He described the refractive peripheral spherules, whose rapid dissolution during artificial digestion led him to believe that they contained very little chromatin. The peripheral ground-plasm appeared as an orange reticulum, bearing a large number of orange granules when stained by the Flemming tricolor technique. Elmassian (1909) considered the peripheral reticulum as a continuation of the central reticulum. The refractive peripheral spherules, according to this investigator, were of a plasto-chromatin nature and were identical with the basophilic granules which he found imbedded in the reticulum of the fixed and stained nuclei. Janicki (1909), on the other hand, reported that the peripheral spherules were completely dissolved during the preparation of balsam mounts, unless dehydration was extremely rapid, when they were but partly dissolved. He was of the opinion that the spherules represented reserve food material for the nuclear elements. In 1910 Mercier observed that the granules comprising the peripheral zone were uniform in size. Kudo (1926) reported that the refractive spherules were of more or less uniform dimensions. They were resistant to staining with Lugol's solution, methylene blue, neutral red, Bismarck brown, and osmic acid. They disappeared on fixation, being replaced by a fine reticulum to which chromatin granules were attached. Morris (1936) described the peripheral zone as granular in the living and fibrous in the fixed state. He says (p. 232), "The peripheral zone contains granules which are highly refractive in living and chromatic in the fixed state." In this interpretation he agrees with Elmassian and disagrees with Janicki and Kudo. Sassuchin (1936) observed that the peripheral granules could be divided into two groups depending on their reactions to stains. Whether or not he means here that the spherules can be distinguished, or that the granules are of two kinds is not clear. At times during the life cycle the peripheral granules were found by Sassuchin to disappear.

Endosomes.—According to Schubotz (1905) there were a number of bodies, varying in size, shape, and number, which lay between the peripheral and central zones. They were not always visible in living nuclei. Spherical in shape, they measured from 2 to 5 μ in diameter. In Flemming triple preparations they were stained a light red. The rapid destruction of the endosomes during artificial digestion experiments indicated a small chromatin content. According to Elmassian (1909) the endosomes were composed partly of chromatin and partly of plastin. He

observed the very considerable variations in the shapes of the endosomes and attributed it to active movements of the nucleus. Janicki, in the same year, expressed the opinion that the endosomes underwent a growth period during the interphase, when chromatin material originating in the central region was deposited on them. In larger endosomes Janicki found transparent vacuoles. Kudo (1926) found the endosomes to be irregular in outline and usually more or less rounded. They were less basophilic than the peripheral granules. Sassuchin (1930) contended that the endosomes were nucleolar in nature. Morris (1936) observed that the endosomes were formed during the early interphase from the peripheral chromatin granules. He described them as hollow bodies with a more lightly-staining core and a more basophilic shell. In number they approximated the number of chromosomes, varying between 12 and 18 or more. The endosomes were destroyed during the late interphase, forming many small spheres and granules. Sassuchin (1936) observed that the endosomes varied a great deal in number, size, and structure, sometimes appearing to be spherical, sometimes oval, and again composed of a series of small spherules.

Central region.—The central region was first described by Bütschli (1878) as a cavity, probably filled with liquid, which sometimes contained a dark body. Schubotz (1905, p. 18) stated, "Schon an frischen Kernen lässt sich in diesen Centrum bei starker Vergrössung ein feines Wabenwerk wahrnehmen." The central region was a weak gray or yellow in Flemming triple preparations. During the course of his artificial digestion experiments almost everything in the nucleus was dissolved by the reagents except a few granules grouped in the center of the nucleus. These granules were specifically stained by Delafield's haematoxylin, and were all of the chromatin in the nucleus, according to Schubotz. Diametrically opposed to this opinion was that of Elmassian (1909), who considered the peripheral and central ground-plasms as continuous, and composed entirely of achromatic material. Janicki (1909) described the central region as an area containing many delicate granules, which he believed to be chromatic in nature. He also mentioned an eccentric karyosome which lay in the central region, and, according to his illustrations, was comparatively large in size. Mercier (1910) disputed the presence of a karyosome. Kudo (1926) found that the central region was more coarsely reticular than the peripheral zone, and was usually lacking in chromatin granules. No karyosome such as that described by Janicki could be found, but he did see an occasional small basophilic granule in the center of the central region, surrounded by achromatic substance. Sassuchin (1930) believed that he could see the karyosome described by Janicki in the living nucleus. Morris (1936, p. 232) states,

"Within this granular (peripheral) zone is a central reticulum which is clear in the living and densely reticular in the fixed nuclei, and in which a dot-like karyosome may sometimes be seen." This karyosome is not identical with the structure of that name described by Janicki, but appears to be the same as the central granule mentioned by Kudo. Sassuchin (1930) describes a cellular structure for the central region. A karyosome was found in some nuclei. This structure, also, appears to be identical with the central granule of Kudo.

B. KINETOPHASE

Mercier (1908-1910) was one of the first to give an account of karyokinesis in *E. blattae*. According to his observations the first indication of approaching nuclear division was the destruction of the endosomes and the formation of a number of fine chromatic granules. These were arranged in a longitudinal series on an achromatic ribbon which was formed in the central region. From the long beaded strand a more or less homogeneous ribbon which was uniformly basophilic was developed. Until this time there were no visible changes in the rest of the nucleus. Through fragmentation of the long "spireme" 4, 5, or 6 pseudochromosomes were formed. Because of the variation in number of these structures, Mercier came to believe that they could not be true chromosomes, hence his term pseudochromosome. As these pseudochromosomes began to migrate toward the poles the nucleus began to elongate. Ultimately a constriction across the longitudinal axis separated the two daughter nuclei. During reconstruction after division the pseudochromosomes became clumped in the center of the nucleus forming a reticulum from which the endosomes were derived.

Elmassian (1909, p. 52) remarked concerning the description of division offered by Mercier, "D'après cette description il nous es difficile de voir la un division mitotique, il s'agit tout simplement d'un changement du noyaux dan lequel les quelques variations de la chromatine ne sont peut-etre autre chose que la modification du noyaux au stade végétativ dont il a été question plus haute." Thus relegating Mercier's observations to changes in the interphase, Elmassian described the reconstruction of the nucleus immediately after division. This was the only stage of karyokinesis that he had seen. In the recently divided nuclei the membrane was appreciably thinner than usual. The whole nucleus was at first filled with fine chromatic granules which gradually aggregated to form the definitive endosomes.

Janicki (1909) offered a contrasting interpretation of karyokinesis. He believed that he had seen two kinds of division. An amitotic type division involved elongation of the karyosome and its subsequent division,

followed by a simple constriction of the nucleus, dividing the peripheral zone and central region into two parts. Elongation of the karyosome likewise initiated mitotic division. It first elongated at right angles to the long axis of the nucleus and later rotated through 90 degrees to coincide with the nuclear axis. A spindle was formed from the karyosomal material. There were more than 6 chromosomes which migrated to the poles of the nucleus without going through an equatorial plate stage. At anaphase they became arranged at the poles of the constricting nucleus, where they formed a rosette, surrounded by transparent karyolymph. The polar clump of dedifferentiating chromosomes became twisted and irregular and finally formed a mass of chromatin at the poles of the nucleus. Nuclear constriction was not completed until the chromosomes were at least partially dedifferentiated. Janicki reported that it required 15 minutes for nuclear constriction to occur in a dividing nucleus observed in life.

Kudo (1926), who, like Janicki, observed division in living and fixed amoebae, reported that just before division began the refractive peripheral spherules began to undergo a very marked Brownian movement. As the living nucleus elongated the clear central region also became oval, and then bilobed as the nuclear constriction began. The peripheral spherules were concentrated in the center of the nucleus as the clear central material migrated to the poles, where, as can be seen clearly in his figures, there remained a small area free from the spherules. This clear area appears to be the divided central region. A long intradesmose connected the two daughter nuclei until the constriction cut through it. One division required 10 minutes while another took 60. One was followed immediately by cytokinesis, and the other was not. In fixed and stained nuclei the early stages of division were characterized by an elongation of the central region and enlargement of the peripheral chromatin granules. These migrated toward the central region and formed several lines, extending from one pole to another, investing the central region. They appeared to increase in number, possibly by division, at this time. Then they were grouped in two masses which moved toward the two poles where they were linked together in rod-like structures. Nuclear constriction separated the two daughter nuclei, after which the chromatin bodies assumed a rounded compact form and were scattered over the achromatic network.

Morris (1936) described mitotic division for *E. blattae*. According to him the first indication of approaching division was the disruption of the typical concentric arrangement of the nucleus and destruction of the endosomes. This produced a sponge-like reticulum which carried chromatin in the form of net-knots. As the nucleus began to elongate

fine filiform chromosomes emerged from the reticulum. These were stained but lightly and were masked by the reticulum up to this time. As the chromosomes migrated to the poles, the nucleus continued to elongate. The chromosomes formed a clump at the poles as the constriction of the nucleus began, after which they became ragged and vesicular, losing their identity in the formation of a new central region. The chromatin spherules derived from the peripheral region were located at the antipolar end of the telophase nucleus, but migrated around the developing central region, thus reestablishing the concentric arrangement typical for the interphase nucleus.

IX. THE NUCLEUS IN LIFE

EVEN UNDER low magnifications the nucleus is very prominent in *E. blattae* because of its large size and highly refractive nuclear membrane. It is usually about $20\ \mu$ in diameter but may be appreciably larger in some cases. A number of nuclei between 20 and $25\ \mu$ in diameter were seen. The largest nucleus recorded in which no indication of parasitism or abnormalcy could be found was just over $30\ \mu$ in diameter. When parasitized by *Nucleophaga* or when undergoing degenerative changes the nucleus increases in size, sometimes reaching almost double normal size. During the early stages of the development of *Nucleophaga* the nucleus remains about normal (Fig. 56), but as the later stages are reached hypertrophy of the nucleus begins. The nucleus appears to reach an ultimate size of nearly twice its normal diameter.

The thickness of the nuclear membrane appears to vary from just less than $1\ \mu$ to about $2\ \mu$. The delicate cytoplasmic membrane investing the nuclear membrane described by Bütschli (1878) was not seen. The most careful search for any indication of a bilamellar structure of the nuclear membrane such as that described by Schubotz (1905) has remained unsuccessful. In bright and dark field illumination the nuclear membrane remains clear and hyaline, without any sign of division. Occasionally one or several bright lines can be seen in bright field, but from their reactions to focussing and shifting of light they seemed to be diffraction images with no structural basis. A beak-like projection at one pole of the nucleus is common. This has been observed by all previous investigators, and generally interpreted as a remnant of the internuclear strand connecting daughter nuclei during division since Janicki (1909) first suggested this explanation. As Janicki observed, the membrane is thinner at this point. The projection appears to gradually decrease in size, with an accompanying increase in the thickness of the membrane. Ultimately it remains as a small, rounded prominence on the nuclear membrane.

The most conspicuous element of the peripheral zone is the large number of highly refractive peripheral spherules (Fig. 38) which are suspended in the almost transparent karyolymph. They vary greatly in size and number in different nuclei, but are comparatively uniform in size and regularly distributed in any one nucleus. The spherules are collected at one side of the nucleus in most cases. This appears to be the result of a slow migration of the spherules through the ground-plasm during nuclear reconstruction following division. Nuclei with the spherules distributed equally throughout the peripheral zone are quite rare. Observed in white light, the spherules have a slightly yellowish tinge. In bluish light they are greenish.

Kudo (1926) reported that the spherules were less numerous in small amoebae with little food in the cytoplasm than in large, well-fed specimens. The same tendency was noticed in the present study. This seems to be explained by the effects of early development leading ultimately to the precystic condition, to be discussed in greater detail subsequently. As the amoebae begin to approach encystment, the division rate increases, and the cytoplasm becomes increasingly clear as the food vacuoles and other included material become less abundant. As a result of the increase in rate of division, the peripheral material of the nucleus becomes greatly diminished (see p. 108). This is true, not only for the peripheral spherules, but for the ground-plasm and endosomes as well. The increase in division rate seems to be the factor most important in bringing about a reduction in the size of the amoebae as well. These facts lead the writer to the opinion that Kudo observed trophic amoebae which were approaching a precystic state. Janicki's suggestion that the peripheral spherules represent reserve food material for the nucleus might be supported by these observations were it not for the fact that the reduction of peripheral spherules seems to be a normal part of the development of precystic forms. It is still possible, of course, that the disappearance of the food vacuoles cuts off the supply of material from which the peripheral spherules are formed. Against this possibility some evidence may be adduced. A study of amoebae from starved cockroaches which contained no food vacuoles and had cytoplasm as clear and transparent as precystic amoebae showed no decrease in the number of peripheral spherules, nor in the size of those present, insofar as could be determined. At least in the larger trophic amoebae, then, the disappearance of food vacuoles is not associated with a decrease in the number or size of peripheral spherules.

Small, inconspicuous granules also occur in the peripheral zone. These bodies, from their distribution and size, appear to be identical with the basophilic peripheral granules. They are never distinct except in nuclei

in which the peripheral spherules are clumped at one side of the peripheral zone. In such cases the small granules can be seen clearly in the opposite side of the zone. Previous investigators have apparently failed to distinguish these granules in living amoebae. Once they have been seen, many of the contradictory statements concerning the staining reactions and relation of the peripheral spherules to the basophilic granules may be understood (see p. 85).

The ground-plasm in which the spherules and granules are suspended is normally in the gel phase, as is shown by the absence of Brownian movement in the peripheral granules. It is not easy to see in the living nucleus, and even in the most favorable conditions appears only as an indistinct outline. In nuclei with the peripheral spherules collected at one pole of the peripheral zone, the ground-plasm can sometimes be seen indistinctly at the other. In such nuclei, even though the substance of the ground-plasm cannot itself be seen, the distribution of the peripheral granules makes it possible to determine the outlines of the peripheral ground-plasm. No Brownian movement was observed in the peripheral granules when the nucleus was compressed, nor during the early degenerative processes occurring in free nuclei outside of the amoebae. Apparently solation of the peripheral ground-plasm occurs only during division.

Lying between the central region and the peripheral zone there is a girdle of large endosomes. These are but rarely visible in life, and, when seen appear as indistinct hyaline bodies without clear outlines. Attempts to determine the differences in nuclei in which the endosomes were visible in life have been unsuccessful so far.

Within the endosomes lies the clear, transparent central ground-plasm. According to Schubotz (1905) this region is reticular in living amoebae. Sassuchin (1936) also believed that he could demonstrate a cellular structure in the central region of living amoebae. The writer has been unable to confirm these reports. With the optical equipment used there was visible a pattern of bright lines through the center of the nucleus. These were visible only in areas covered by the peripheral spherules, and appeared to be produced by diffraction of light through the spherules. Dark field studies, likewise, failed to reveal a reticular structure.

Stages of late nuclear reconstruction were studied in life. At this time the Brownian movement of the peripheral spherules described by Kudo had ceased, and the peripheral region had an uneven distribution of the various elements. The spherules were clumped at one pole, and very gradually moved around the central region. This was the only change that could be followed in life in the material studied.

X. NUCLEAR ACTIVITIES DURING THE TROPHIC STAGE

It is not easy to determine which structures are artifacts and which are truly reflections of structures present in the living nucleus in the case of *E. blattae*. The large number of nuclear elements, some of which are invisible in life but visible in the fixed nuclei and others of which are visible in living nuclei but usually absent in fixed nuclei, serve to complicate the problem. In the following account of the nuclear appearance and nuclear changes during interphase and kinetophase the interpretations made are not explained in all cases. All interpretations were based on the study of the effects of fixatives on the nucleus, and the bases for them are treated in subsequent sections. Detailed and exhaustive accounts of slight variations in appearance due to fixation and staining are not given here. These, too, can be found in succeeding sections. This discussion, then, represents the generalized sum total of the experiences of the writer, critically considered, with the view of describing and interpreting the nuclear activities of the trophic amoeba.

A. THE INTERPHASE

In the nuclei of most organisms the interphase condition is a morphologically static stage during which the metabolic activities are carried out with a minimum of visible changes. The chromatin remains in a dispersed state throughout the interphase, or is centered in a definite endosomal body with a greater or lesser amount scattered between the endosome and the nuclear membrane. This appears to be quite unlike the condition in *E. blattae*. Definite morphological alterations accompany the interphase nuclear activities. When the division rate is high the nucleus has an appearance distinctly unlike that observed in cases where the division rate is low. In hosts in which a large number of division figures were found interphase reorganization was restricted. When the division rate is low the nuclei remain in the interphase condition for a relatively long period of time, and the organization and appearance of the nucleus reflect the long duration of the metabolic activities without nuclear reorganization.

The interphase proper begins when the migration of peripheral elements around the central region produces a continuous peripheral zone around the central chromosomal material, which is at this time negative to the Feulgen reaction. The central region is granular, and in some cases contains a few indications of the old dedifferentiating chromosomes. The endosomes are present as series of basophilic granules or spherules connected by a lighter-staining substance, or as discrete small spherules. The

ground-plasm is filled with a large number of peripheral spherules and granules. The spherules remain clumped at one pole of the nucleus, while the granules are more or less equally distributed throughout the whole peripheral ground-plasm (Fig. 4).

The reorganization of the central region during early interphase entails the disappearance of granules associated with the chromosomes, an almost complete loss of affinity for basic dyes, and a loss of the positive reaction to the Feulgen nucleal test. At the late kinetophase period, the chromosomes are found in a clump at one end of the nucleus, more or less fused together, and already partially or almost wholly lacking in basophilic material (Figs. 1, 2). Nuclei, unless fixed with Flemming, do not show a hyaline body. After Flemming fixation, however, a hyaline body may be found (Fig. 4). This structure, becoming less basophilic as the interphase advances, is characterized by its constant polar position adhering to the central region, and its clear hyaline appearance. It remains for part of the interphase period (Fig. 15). The central region gradually comes to occupy a position in the center of the nucleus, migrating slowly from the pole as the peripheral ground-plasm, carrying peripheral granules with it, moves down and around it. Apparently the central ground-plasm, during this period of development, is unusually dispersed, for it is usually shrunk more during fixation than at later stages, drawing away from the peripheral ground-plasm to a marked degree (cf. Figs. 4 and 9). After the central region has come to occupy a central position, or is approaching it, the central and peripheral ground-plasms usually are connected by radii-like strands of the central ground-plasm (Figs. 5, 6), unless fixed in a fixative which entails little or no shrinkage of the ground-plasm, in which case it is attached to the peripheral region along its whole margin (Figs. 9, 10). The hyaline body is at first slightly basophilic, but as the peripheral material gradually invests the central material the hyaline body becomes less basophilic and stains rather deeply with the acid dyes. Shortly after the central ground-plasm has attained its final appearance and is no longer colored by the Feulgen reaction, the hyaline body disappears, to be seen no more until the late kinetophase of the next division. Insofar as the central ground-plasm is concerned, there are no marked changes in its appearance from this time until the chromosomes begin to appear during the following division. It is at this time either a reticular or amorphous region of acidophilic material, which is occasionally found to contain small granules of more intensely acidophilic substance, especially after fixation in Gilson-Carnoy. Attempts to correlate the appearance of these granules with endosomal appearance have, thus far, been unsuccessful.

At the time that the central ground-plasm is forming in the chromo-

somal clump at the pole of the nucleus, the peripheral ground-plasm, migrating along the nuclear membrane to the pole of the nucleus, contains the deeply basophilic endosomal bodies and the peripheral granules, slightly less basophilic. Ultimately a more or less even coating of the nuclear membrane is formed, and from this time on the peripheral ground-plasm retains its structure and appearance. It is usually reticular, especially after the less vigorous fixatives, but occasionally develops an odd fibrous appearance (Fig. 14), in which the alveolar walls tend to be stretched at right angles to the nuclear membrane. The endosomes likewise tend to become more evenly distributed around the central ground-plasm, but the peripheral spherules lag behind the other peripheral elements, usually retaining their uneven distribution until the next division. Nuclei in which the peripheral spherules are evenly distributed are extremely rare.

Once the typical concentric arrangement of the various nuclear parts has appeared, with the central ground-plasm occupying a central position, surrounded by a sub-equal layer of the peripheral ground-plasm containing spherules and endosomes more or less equally distributed throughout, very little change occurs in the nuclear elements, exclusive of the endosomes, until the next division. These undergo a series of gradual changes which comprise the most striking feature of interphase activities. In the early interphase there is a great diversity of appearance in the endosomal material which appears to be reflected in later stages. The diversity of appearance is such that there can be no doubt that the endosomal changes do not follow a rigid course of development. The variation in endosomal appearance in late interphase stages appears not to be a random one, however, and the writer believes that there is a seriation of indefinite morphological types.

The endosomal development begins during the late kinetophase. At this time endosomal anlage are formed from basophilic endosomal spherules derived from the endosomal substance of the parent nucleus during the early kinetophase stage (Figs. 1, 2, 3). The endosomal anlage appear as strands of endosomal spherules, which are connected by a more lightly-staining substance. Not all of the spherules are fused to form the endosomal anlage at this time, however, and a greater or smaller number of them remain as discrete bodies at the pole of the nucleus lying opposite to the dedifferentiating chromosomes. At first the endosomal anlage tend to lie in straight lines, but after the peripheral ground-plasm has finished its migration around the central region, the endosomal anlage assume characteristic sinuous shapes (Figs. 7, 12). The original beaded appearance of the endosomal strands gradually disappears and the endosomes appear as winding bars of deeply basophilic material. In nuclei

which have undergone a greater amount of differentiation after staining, however, the spherules composing the anlage can still be seen (Figs. 5, 9, 11). The spherules are more distinct in nuclei which have been stained with safranin than in nuclei which have been stained with haematoxylin in these later stages, unless differentiation is carried far beyond the normal point. This indicates that the lighter-staining substance which originally connected the endosomal spherules develops an affinity for haematoxylin at a more rapid rate than it develops an affinity for safranin. The endosomes later lose their beaded appearance even when destaining is almost complete, and at this time show a definite tendency to become shorter and heavier (Fig. 13). During this stage of their development the resemblance of the endosomes to metaphase or late prophase chromosomes of metazoan nuclei is very striking, which probably accounts for the term pseudochromosome which has been used by Mercier (1910) and Morris (1936) to indicate these middle interphase endosomes. It is interesting to observe that the endosomes tend to assume angular shapes after Gilson-Carnoy fixation (Fig. 16), but tend to be spherical after Schaudinn or Flemming fixation (Fig. 17). Zenker fixation, likewise, favors the spherical shape. It appears that most nuclei do not progress beyond this stage but undergo a division before later developments occur. This, then, is the typical interphase condition (Figs. 15, 16). If the division rate is extremely low, later endosomal changes may occur. To what extent these are degenerative and to what extent normal is not known at this time. The endosomes become larger and develop a vacuolated appearance. Even in the middle interphase condition a differentiation of a cortical deep-staining and an inner lightly-staining portion is frequently observed, but in these very late stages the endosomes show this characteristic even more clearly (Fig. 18), and a small central granule of basophilic material can be seen. It is not uncommon for the lightly-staining material in the endosome to become distributed in small vacuoles during further development, which entails a reduction in the number of endosomes and an increase in size. It was thought that this might be an indication of the beginning of the disintegration of the endosomes preparatory to the next division, but since most nuclei divide long before this stage is reached, it seems improbable that it is a regular occurrence.

In one host, especially, and more rarely in several other hosts, the endosomes were sometimes found in a very characteristic orientation. Endosomes in the short rod condition, approaching the cuboidal shape, became arranged in a more or less parallel pattern forming a girdle of endosomes about the nucleus (Fig. 13). The significance of this type of development is not known.

This endosomal development has not entirely escaped the attention of

earlier investigators. Mercier (1910) described part of this process (see p. 34). The process involved, as he described it, the formation of beaded strands which became homogeneously basophilic and assumed sinuous shapes. These strands formed a spireme thread which broke up into pseudochromosomes. The writer has observed several nuclei in which the endosomal substance was almost entirely gathered in one long sinuous endosome. Mercier reported that there were usually from 4 to 6 pseudochromosomes. In the material used for this study there were usually a dozen or more. This may be an indication of a racial difference in the materials studied. At the present time there is no information on the variations which occur in *E. blattae* in different species of cockroaches growing under various environmental conditions, so that no positive evidence can be adduced for this point. The later stages of division described by Mercier are not very clear. Amoebae have occasionally been found which resemble certain of his figures, but it is not as yet completely understood. This might, again, be associated with racial, environmental, or host differences. Mercier, then, has described some of the steps in the development of the endosomes during the interphase, although he believed that they were a part of the division phenomena.

Earlier work of Mercier (1907, 1909) in which the same division cycle was described in practically the same words brought forth a comment from Elmassian (1909) that he believed it probable that Mercier had observed vegetative rather than division phenomena (see p. 34). Elmassian appears to have noted some of the endosomal changes mentioned and interpreted them as vegetative in nature. He is thus the first to hint at interphase nuclear changes. Janicki (1909) also mentioned endosomal variation and described the growth of the endosomes during interphase. Morris (1936, p. 233) mentions the variation in endosomal appearance and says, "They exhibit a gradual change, from late telophase to early prophase, seeming to alternate in period of activity with the true chromosomes." Unfortunately he does not elaborate on this statement insofar as the changes he had noticed are concerned.

The endosomal activity is quite unlike that found in most of the other members of the genus *Endamoeba*. In the *Endamoeba* described by Kirby (1927) from termites a more or less similar nuclear appearance during interphase was noticed, although he did not describe any trends of endosomal changes similar to those mentioned here. Most striking was the occurrence of the elongate sinuous endosomes in *E. disparata*, very similar in all respects to those found in *E. blattae*. In *Euglypha* sp. Bělář (1926) described endosomes which were, in many respects, similar to the elongated endosomes found in *E. blattae*. These, however, lay in the center of the nucleus instead of the periphery. They were broken

down during early division stages, and the chromosomes were formed from peripheral material and not from endosomal material. This appears to be a case in which the arrangement of the nuclear material is the exact reverse of that observed in *E. blattae*, although the conditions appear to be analogous in most respects.

The functional significance of the endosomal alteration during interphase is not understood completely. The changes suggest that the endosomes may be extremely active during the metabolic period. Morris (1936, p. 245) has considered the possibility that the peripheral chromatin of *E. blattae* represents trophochromatin. He says: "A plausible explanation of the behavior of the peripheral chromatin of the adult *E. blattae* is not easy to reach. Its apparent quiescence during the mitotic portion of the nuclear cycle and notable activity during the interphase seem to set this species in a group by itself. The nearest approach to this phenomenon appears to be the case of the opalinid, *Zelleriella*, described recently by Chen (1932), but even this does not present a perfect analogy. Metcalf (1909) and, later Tönniges (1919) and Bělař (1926) suggested the similarity of the large chromatic bodies in the opalinid nucleus to the macronuclear material of such infusoria as *Paramecium*, and this may well be so, regardless of the fact that these writers failed to describe the chromosomes correctly and therefore misinterpreted much of what they saw.

"If the same analogy holds true for the trophochromatin of *E. blattae*, although this must still be considered hypothetical, then it may well be that the peripheral chromatin of all the Endamoebae should be considered in this way, for the pseudochromosomes of *E. blattae* give every indication of being homologous to the trophochromatin of other members of the genus."

The writer wishes to subscribe to the opinion of Morris. As will be seen in later sections of this study, there is a great difference in the staining reactions of the peripheral and central "chromatin" of the nucleus of *E. blattae*. This, with the difference in their reaction with fixatives, appears to indicate definite chemical differences (see p. 87 ff.). It is probable, although yet hypothetical, that the endosomes contain a nucleoprotein, while the central region contains, at division stages, nucleic acid, and during the interphase a nucleoprotein, which, in chemical composition or in physical state, is distinct from the nucleoprotein of the endosomes. The paradoxical situation in which the most prominently basophilic nuclear elements, the endosomes, do not show a direct genetic relationship to the chromosomes is not unlike the situation in *Amoeba proteus* as described by Chalkley (1936). At the present time no definite conclusions can be drawn, but it becomes increasingly possible that, as in the ciliates,

there is a segregation of trophic and kinetic chromatin which may express itself in the nuclear structure of the organisms and in the relationship of the nuclear elements composed of "chromatin" to the true chromosomes.

B. THE KINETOPHASE

Kinetophase changes may begin at any point in the interphase cycle, depending on the division rate. The chromosomal cycle appears to be the same, regardless of the division rate, but the activities of the peripheral material depends to a certain extent on the length of the interphase preceding the division and the appearance of the endosomes at the time division begins.

The central region of the late interphase nucleus is composed of a reticular or homogeneous region in permanent mounts, in the center of which a centriole can be seen in many nuclei. At this time the ground-plasm is more or less acidophilic, depending to some extent on the fixative, and invariably negative to the Feulgen nuclear test. The first noticeable step towards division is observable in the central region, which becomes somewhat more reticular, and begins to show slight affinities for the basic dyes. At this time a slight tint of violet is observed in nuclei subjected to the Feulgen reaction. This positive reaction with the Feulgen reagents occurs diffusely in the whole ground-plasm of the central region. This is followed by the appearance of delicate granules, which are especially well demonstrated after fixation in Gilson-Carnoy and Flemming (Figs. 20, 21). These granules appear to be more basophilic than the surrounding ground-plasm, and in rare instances seemed to be more intensely colored by the Feulgen reaction than the ground-plasm. This last observation is not made with a great degree of certainty, however, as the reaction is too light at this time to make comparisons certain. The granules are soon afterwards arranged on strands which lie twisted and coiled together in the mass of central ground-plasm, which, at this time, becomes noticeably smaller in amount. These strands, like the granules, show a greater affinity for basic dyes than does the surrounding ground-plasm and appear to react more intensively with the Feulgen reagents. The strands assume a beaded appearance as the granules are collected along them. At this time the centriole, when visible, is double (Fig. 21). It is at this time that the nucleus suddenly enters into a "dedifferentiated phase" which disrupts the normal concentric arrangement of the nuclear elements. The peripheral material is greatly altered in appearance as the ground-plasm becomes very irregular in distribution, and the endosomes are broken down into small spherules (Figs. 22, 23). At this time it becomes impossible to differentiate the central region, and the whole nucleus becomes filled with a

spongy mass of material with the endosomal substance distributed at random throughout the nucleus. This period appears to coincide with the period of active Brownian movement described by Kudo (1926), who watched living nuclei in the process of division. The endosomal spherules and the peripheral ground-plasm gradually come to occupy the central part of the nucleus, investing the central region, which becomes visible once again beneath the girdle of peripheral material (Figs. 24, 25, 26). The central region is now distinctly elongated or bilobed, and the chromosomal strands are much more distinct, while the centrioles show a distinct tendency to be much farther apart than before the "dedifferentiated phase." The nucleus is usually beginning to elongate at this time. The most careful search has failed to show any stage comparable to a metaphase. As soon as the peripheral material has arranged itself in a girdle about the central part of the nucleus, the chromosomes, still twisted and coiled, begin to migrate toward the poles, and appear protruding from the edges of the peripheral girdle. The central ground-plasm is not entirely gone at this point, and usually assumes a bilobed appearance during the first part of the migration. It is during this migration that the chromosomes undergo their final development and appear as homogeneous strands, or, in some cases, as comparatively heavy strands of light-staining substance containing small granules of basophilic material in them. As the nucleus begins to constrict, or, sometimes, before constriction begins (Figs. 27, 28, 29) the chromosomes have completed their migration to the poles, and may be seen in a rosette at the poles of the nucleus. During the last portion of their movement to the pole they assume a characteristic V-shape, similar to that of chromosomes during anaphase in metazoan mitosis. At this time they react very distinctly to the Feulgen technique, and show some affinity for basic dyes. The endosomal substance, gathered in small spherules after the "dedifferentiated phase," remains very intensely basophilic, much more so than the chromosomes, but does not react with the Feulgen reagents. Nuclear constriction appears to occur rather more slowly than the changes preceding it, and long intradesmoses sometimes persist for some time after division appears to be complete in other respects. As the nucleus constricts, and the chromosomes reach their polar position, the peripheral material comes to occupy the center of the elongated nucleus. The endosomal spherules and the refractive peripheral spherules tend to lie in straight lines at this time (Fig. 29), although the linear arrangement is frequently quite irregular, and at least partially disrupted by the tendency of the endosomal spherules to form a coarse reticulum (Fig. 30). The spherules of refractive substance so characteristic of the living nucleus cannot be studied advantageously in permanent mounts made

from material fixed with compound fixatives, but were observed in amoebae fixed with formalin. In these nuclei the tendency of the peripheral spherules to lie in straight lines during the constriction of the nucleus was very marked, even more so than was the case with the endosomal spherules. After nuclear constriction is complete the two daughter nuclei reorganize themselves into typical interphase nuclei.

During the reorganization of the interphase nuclei from the daughter nuclei, the endosomal spherules form in lines, and become connected by a light-staining substance, described with the interphase nucleus. These endosomal anlage gradually become homogeneous in their staining reactions, and migrate with the peripheral ground-plasm about the central region, which gradually comes to assume its definitive central position.

In safranin, particularly, the chromosomes appear as a row of basophilic granules arranged on an acidophilic strand. The granules are conspicuous during the time that they form a rosette at the poles (Fig. 28), but as the interphase reorganization occurs the granules become less prominent and ultimately disappear entirely. As the clump of chromosomes begins to undergo its transition into the central region, a mass of hyaline material appears at the pole of the nucleus. This is quite basophilic at the time that the granules are visible (Fig. 4), but afterwards gradually loses its affinity for basic dyes, and ultimately stains almost exclusively with the acid dyes. As the hyaline body appears, the chromosomes fuse together, lose their individuality insofar as can be determined visually, and form the central ground-plasm of the daughter nuclei.

The endosomal material lying in the constricted region of the new daughter nucleus is less easily followed. The endosomes are destroyed in the early kinetophase, and are not present as such during division. In their place a large number of small endosomal spherules are found. The method of endosomal destruction and the relationship of the old and new endosomes to the endosomal spherules appears to have been a source of much difficulty in descriptions of the division of *E. blattae*.

Kudo (1926, p. 147) says, "When the nucleus starts to divide the chromatin granules become larger in number and size. . . . The chromatin granules become grouped into a number of somewhat larger bodies, spherical or rounded-oval, which become arranged in several lines between the poles over the achromatic reticulum." This would indicate that there was a fusion of the peripheral granules to form the endosomal spherules. Morris (1936, p. 233) differs in his interpretation, saying, "They (the late interphase endosomes) appear to be hollow, or composed of lightly-staining material surrounded by a more heavily-staining zone. This condition terminates with a breakdown into spheres and granules as

the prophase approaches." Earlier he says (p. 233), "During the late telophase-early interphase period the granules of peripheral chromatin are usually in the form of connected series like strings of beads. These coalesce to form large chromosome-like bodies or pseudochromosomes, intermingled with small dense chromatic granules." The relationship of the material formed from the old endosomes to the new endosomes is not made clear. If the peripheral chromatin granules form the new endosomes, the fate of the old endosomal material is still unexplained.

Certainly the two most probable methods of endosomal disruption are gradual disintegration by vacuolization and loss of basophilic material, or disruption into endosomal spherules directly. During the late interphase period the endosomes are fewer in number and larger in size. These larger endosomes are vacuolated, and in some cases show by irregular contours that they may be produced by fusion of smaller elements. The development of vacuoles may possibly be taken as a sign of loss of basophilic material by slow dissolution or chemical change resulting from use. Fusion in itself may be a sign of early disintegration. In some of the larger endosomes there is no sign of fusion, indicating that the endosomes may have grown directly before beginning, or while in the process of, vacuolation. These observations seem to indicate that the destruction of the old endosomes simultaneously involves fusion, vacuolation, and dissolution of the basophilic material. On the other hand the nuclei found in hosts showing a high division rate show no indication of vacuolation of endosomes. In such nuclei the endosomes seem to break down directly into smaller basophilic elements, and since they appear at this time, the endosomal spherules are the most probable products. Indeed, during the early kinetophase "dedifferentiated stage," the formation of the spherules from endosomes can be seen directly. A combination of the two methods, with the emphasis on vacuolation when the division rate is low, and on a general physical disruption when the rate is high, seems to be the most satisfactory explanation with the data now at hand.

In case the endosomal material is largely dissolved the endosomal spherules of the dividing nucleus must be developed from some existing nuclear element other than the endosomes. This seems to devolve on the peripheral granules, which, as Kudo says, increase in size and become grouped together to form the larger spherules. It has not been possible to demonstrate fusion of the peripheral granules, and to what extent they enter into the formation of endosomal spherules has not yet been determined.

Once the endosomes have disintegrated and the endosomal spherules have formed, the nucleus enters the so-called "dedifferentiated phase."

This is characterized by the coarse reticulum carrying a large number of basophilic elements, completely surrounding and obscuring the central region. The probable significance of this has never been completely understood. Morris suggests, and with the probability of being correct, that it is associated with the Brownian movement of the peripheral spherules observed by Kudo during division of living nuclei. The fact that the reticulum formed by the peripheral ground-plasm is so irregular at this time may possibly indicate that there has been a shift from gel to sol phase in the material comprising the peripheral region.

The spherules then become arranged in rows extending from pole to pole of the elongating nucleus, as described by Kudo. These rows are superficial, lying around the central region, as can be plainly seen in rare favorable cases where the peripheral material does not wholly obscure the central region (Figs. 25, 26). The constriction of the nucleus divides the endosomal spherules between the two daughter nuclei, but there is no indication of a true quantitative division. No mechanism for accomplishing an even distribution has been found, unless the tendency toward forming rows, extending almost from one pole of the constricting nucleus to the other, be considered as having this function.

The breakdown of the endosomal substance and the formation of new endosomes with each division of the nucleus is an extremely interesting phenomenon. If the peripheral basophilic material is considered as trophochromatin, it acquires some significance. In the macronucleus of several ciliates reorganization bands have been described. Turner (1930), Kidder (1933), and Summers (1935), for example, have described such phenomena in *Euplotes patella*, *Conchophthirius mytili*, and *Aspidisca lynceus* respectively. Occasionally this is accompanied by the discarding of a part of the macronuclear chromatin, as described by Kidder for *Conchophthirius*. This may be paralleled in *Endamoeba blattae*, as Kudo (1926, 1939) points out. In the division of a living nucleus Kudo observed the formation of a small bulb in the connecting strand, which may be compared to the chromatin cast out of the macronucleus of *Conchophthirius*. The writer has observed similar bulges in the connecting strand extending to daughter nuclei in fixed preparations. How frequently or regularly they occur is not yet known, but it seems certain that they are not of universal occurrence, for in many division figures the bulb is wholly lacking.

The parallelism of the endosomal changes of *E. blattae* and the macronuclear changes of these ciliates is in many ways most striking. The ciliate reorganization band appears to involve a shift from gel to sol phase in the region of the clear solution plane. In *E. blattae* there is a shift from gel to sol phase in the peripheral ground-plasm, which

apparently accompanies the breaking up of the endosomes. The bulb is composed entirely of peripheral material, since the peripheral material comes to occupy the central part of the constricting nucleus. Thus the endosomal material must be represented in the discarded material, and appears in it in fixed preparations. But the significance of this parallelism cannot be determined at the present time. The mechanisms involved are quite dissimilar. In addition, there is some uncertainty which we necessarily feel concerning the question of whether the endosomal substance can be reasonably called trophochromatin, at least in the same sense as can the ciliate macronucleus. The writer, however, does wish to emphasize the fact that the parallelism in the activities of the peripheral chromatin of *E. blattae* and macronuclear organization of some ciliates serves to support, in some degree, the idea that the peripheral chromatin of *E. blattae* may be a type of trophochromatin.

When the constriction is completed the daughter nuclei have a very characteristic appearance (Fig. 31). At the pole lie a number of chromosomes in the form of a rosette. The whole antipolar region is packed with the endosomal spherules and the peripheral spherules, which, like the former, were passively distributed to the two daughter nuclei in about equal numbers. The concentric arrangement is restored by the migration of the peripheral material about the developing central region. The endosomal spherules begin to fuse together during this migration, forming the endosomal anlage (Figs. 1, 2, 3), composed of the intensely basophilic endosomal spherules connected by strands of more lightly-staining material. The endosomal material sometimes appears to precede the peripheral ground-plasm in the migration about the central region. As the ground-plasm reaches the polar end of the nucleus, completely investing the central region, which, by this time, has completely or almost completely lost its ability to react with the Feulgen reagents, the interphase begins.

Formation of new endosomes appears to involve a fusion of the endosomal spherules rather than the peripheral granules, as suggested by Morris. The early endosomal anlage appear to be composed of basophilic structures too large to be identified as peripheral granules, and more basophilic than the granules. Indeed, it is possible to decolorize the granules entirely, while leaving the endosomal spherules still quite deeply stained (Fig. 5). It seems possible that at least some of the endosomal spherules are partly formed from the peripheral granules during the early division stages, and also that the gradual development of a homogeneous staining reaction in the endosomal anlage entails an accumulation of the peripheral granules in the lightly-staining portion of the anlage, but this has not been definitely demonstrated. It is certain, how-

ever, that there is an increased amount of basophilic material in the endosomes, developed during early interphase, which may quite possibly arise from the lightly-staining substance composing the connecting strands of the anlage, or may be obtained from the peripheral granules.

Division, as it occurs in *E. blattae*, is quite unlike that described for *Entamoeba histolytica* by Kofoid and Swezy (1925) or by previous investigators for that species. The work of Cleveland and Saunders (1930) only serves to make the difference between these two species of amoebae even more striking. The close similarity of the division of the termite Endamoebae, *E. disparata* and *E. simulans*, described by Kirby (1927) supports the idea that these organisms are very closely related and are rather distantly related to other forms of parasitic amoebae.

The termite amoebae and *E. blattae* present a very characteristic type of division, entailing the absence of a metaphase plate, and the presence of a comparatively large number of chromosomes which appear in the center of the nucleus as beaded strands. Kirby did not use the Feulgen reaction, so that the question as to whether or not there is a variation in the demonstrable nucleic acid of the nucleus of *E. disparata* or *E. simulans* during division, similar to that observed in *E. blattae*, is not known. The activities of the endosomes during division are similar in these forms also. They are broken down at the time of division and do not appear to contribute directly to the formation of the chromosomes.

There appears to be a growing tendency toward the separation of the several types of Endamoebae into subgroups. Morris (1936) suggested that the genus Endamoeba be divided into three subgenera. One of these, sub-genus Endamoeba of which the type is *blattae*, is suggested for the cockroach and termite amoebae. Two other subgenera, Placoidia with type *E. (P.) minchini* and Poneramoeba with type *E. (P.) histolytica* were used for the other types of amoebae occurring in the genus Endamoeba. The writer agrees with Morris in feeling that some sort of a separation is necessary. However, in spite of the ruling of the International Commission on Zoological Nomenclature (Opinion 99) that Endamoeba and Entamoeba are synonyms, the writer feels that it would be more logical to retain the names Endamoeba and Entamoeba, and limit the former to the cockroach and termite amoebae, and other forms that present the same characteristics of a thick-walled nucleus with a number of prominent endosomes and nuclear division lacking a metaphase plate stage. Under the latter the two subgenera suggested by Morris, Placoidia and Poneramoeba, could be used. This opinion is based on the fact that in general characteristics and in the activities of the nucleus during interphase and division, there is a more fundamental difference between the Endamoeba type and the other two than is consistent with a subgeneric

grouping. In view of the fact that later evidence has appeared, which shows quite clearly the difference between the types of amoebae, and since it seems undesirable to alter the name of the human parasites which have the great mass of literature concerning them, this division seems convenient and helpful. Dobell (1919) and Kudo (1931, 1939) have advanced this suggestion. Kudo (1939, p. 312) expresses this point of view very aptly, saying, "The generic differentiation is based upon morphological characteristics of the nucleus. Summary No. 99 of 'Opinions Rendered' by the International Commission of Zoological Nomenclature (1928) holds that *Entamoeba* is a synonym of *Endamoeba*; in the present work, however, *Endamoeba* and *Entamoeba* are separated, since the two groups of species placed under them possesses different nuclear characteristics and since it is not advisable to establish another generic name in place of *Entamoeba* which has been so frequently and widely used throughout the world."

XI. EFFECTS OF FIXATION ON THE NUCLEAR ELEMENTS

WHEN A NUCLEUS is exposed to reagents which precipitate the substances which compose it in a form sufficiently insoluble so that technical procedures, such as sectioning and staining, can be performed, it goes without saying that many profound changes of a chemical and physical nature have ensued. A comparison of the living nucleus with the fixed and stained nucleus in permanent mounts emphasizes the importance of these reactions. In the case of *E. blattae* it appears that the changes accompanying fixation and staining are more complicated and numerous than is the case with many other types of nuclei. To make them more difficult to understand, they vary significantly with different fixatives. Generally speaking, the effects involve the solution and total degeneration of the peripheral spherules, and a great increase in the visibility of the peripheral granules, the endosomes, and the central ground-plasm. Other differences of a more detailed nature are noticed when a close comparison is carried out.

The utility of any fixed and stained preparation is in proportion to the adequacy with which one can interpret the results in terms of the living nucleus. Until the question of artifact formation is understood no thorough interpretation is possible. Change of visibility in the nuclear elements is helpful, for it makes a more complete view possible, if it is certain that the final image is not too aberrant. Indeed, were it not for the changes in visibility there would be no need nor use for permanent preparations. It seems probable that any structure which appears after

fixation with several different fixatives, which shows a constancy in its position, and which is consistent in its staining reactions actually exists in the living nucleus, either as a distinct structural unit or as a region of differentiated protoplasm. Before these structures, visible only in fixed nuclei, can be interpreted in terms of their function, however, it is necessary to know their exact relation to the structures found in the living nuclei.

In order to bridge the gap between the living nucleus and the nucleus as seen in the fixed and stained mounts a series of observations were made on the process of fixation and the effects of dehydration and staining. Every fixative used for important and extensive observations and all the reagents which composed those fixatives were studied in the same way. The living amoebae were compressed slightly beneath a cover slip and studied under oil immersion before, during, and after the application of the fixing reagent. In order to facilitate a comparison with permanent mounts the nucleus was stained, after appropriate washing, with acetocarmine or methyl green. In all cases observation of the nuclei, from the beginning of the experiment until its conclusion, was continuous. The results of this preliminary study were followed by a careful comparison of the immediate effects of fixation with those occasioned by dehydration and staining. Knowing what the effects of the fixative were, the differences between the nuclei immediately after fixation and those in permanent mounts represented the effects of the later technical processes. With the hope of gaining some information as to the relative solubility, and through this, of the probable nature of the various precipitated elements, two types of permanent mounts were made with each fixative. One set of sections and smears was stained with a minimum amount of contact with water. Another set was washed for long periods of time in running water. The differences observed represented solution of precipitated nuclear elements in water, and an idea of the relative solubility of the various nuclear structures after treatment of the fixing reagent was obtained.

A. SIMPLE, UNCOMBINED FIXATIVES

Ethyl alcohol.—It is generally observed in most discussions of technique that different dilutions of alcohol cause different effects during fixation. Low grades of alcohol are said to cause much more serious malformation of the cell during fixation than absolute alcohol. In order to observe these differences, absolute alcohol, 70 per cent alcohol, and 30 per cent alcohol were used. The absolute alcohol was used at room temperature and warmed to 45-50° C. The lower grades were used only at room temperature.

The effects of alcohol as a fixative have been studied by several previous investigators. Baker (1933) describes a great deal of shrinkage, both nuclear and cytoplasmic. He also reports a tendency toward the pushing of cell and nuclear contents to one side of the cell. This was especially marked in the peripheral parts of the tissue. Strangeways and Canti (1927) report that there is no very great disturbance of cell morphology during fixation. It is interesting to note here that Baker's study was of tissue sectioned and stained after alcohol fixation, and Strangeways and Canti (1927) observed the process of fixation in isolated cells studied under dark field illumination.

With regard to the physico-chemical effects of alcohol as a fixative, there are several notes available. Fischer (1899) reports that albumins are precipitated in a form which is insoluble in water, while nucleic acids are soluble. Mann (1902) reports that nucleo-albumins and nucleins are insoluble after alcohol fixation. The precipitation of proteins, called "denaturation," is explained by Baker (1933) as one in which the protein linkages are affected, altering the solubility of the compounds and their affinity for acids and bases, without appreciably affecting their chemical nature.

In the study of the immediate effects of fixation the alcohol was applied to the edge of the cover slip and drawn under it rapidly with a piece of paper toweling applied to the opposite edge. It must be noted here that some dilution of the alcohols must have occurred during this. The small amount of water and the large amount of alcohol used indicates that the dilution was not great. Fixation with alcohol was slow, requiring from 2 to 3 minutes for the visible alterations of nuclear appearance to cease, although the animal was killed almost instantaneously. Strangeways and Canti (1927) reported that alcohol fixation was instantaneous. At the moment of fixation the nuclear membrane became thinner, probably at least partially the result of the lifting of the cover slip by the advancing wave of the fixative. No other visible change affected the nuclear membrane. No change in the position, shape or size of the peripheral granules was observed, but they became much more refractive. The peripheral ground-plasm became much darker and was precipitated in very fine granules. There was no effect on the peripheral spherules. This failure of the alcohol to dissolve the peripheral spherules during fixation seems to indicate that the belief of Janicki (1909) that the spherules were dissolved by the alcohols during dehydration is probably incorrect. The endosomes, sometimes partially hidden by the peripheral spherules, became slightly more refractive the instant that the alcohol struck the animal. After this there was no further visible change in the endosomes. The central region, transparent in life, underwent the most

striking series of alterations. A number of fine strands appeared first, several seconds after the alcohol came in contact with the amoeba. These became more prominent, and about a minute after fixation had begun a number of fine granules appeared on the strands. Several moments after the strands became prominent the central ground-plasm began to precipitate. The precipitate was in the form of extremely small particles which gradually increased in numbers and ultimately obscured the strands and granules. When fixation occurred in the proper kinetophase stage a hyaline body could be observed. When this homogeneous structure was noticed at one pole of the nucleus, it was accompanied by a number of delicate granules in the ground-plasm of the central region which appeared just before the ground-plasm was precipitated. These were not observed in interphase nuclei.

The most characteristic phenomenon observed in alcohol fixation was the gradual appearance of the various nuclear elements. In almost every other fixative tried fixation was instantaneous. In every experiment the elements formed slowly and in all cases the various nuclear elements appeared following the same temporal sequence. The addition of methyl green to the fixed nucleus showed that the peripheral granules were basophilic, the endosomes somewhat more so, and the hyaline body somewhat less so. After some time the whole nucleus became lightly stained with the green dye.

While still lying in the alcohol the nuclei were very accurately and well preserved. Shrinkage was much less than had been anticipated, especially in the lower grades of alcohol. The nuclear elements appeared to be preserved in approximately natural conditions, and there was no observed tendency of the cell or nuclear contents to draw to one side of the cell. The results of observations on the immediate effects of fixation appear to confirm Strangeways' and Canti's (1927) report that alcohol does not cause any striking alteration of form.

It was only in permanent preparations that aberrations and other indications of poor fixation were found, as will be noted below. The nuclear membrane was homogeneous and about the same regardless of the percentage of alcohol or temperature. It was acidophilic, and resisted staining with basic dyes. The membrane was greatly wrinkled in slides fixed in 70 per cent alcohol, due to shrinkage of the nucleus. In many cases the uneven shape was so disturbing that the nuclear elements within could not be studied satisfactorily. This effect was much less intense in absolute or in 30 per cent alcohol. The low grade of alcohol, insofar as nuclear shape and amount of shrinkage was concerned, was superior to 70 per cent, and very little inferior to absolute. Few nuclei were misshapen, and, based on comparative measurements, seemed to

show but little shrinkage. There was no indication that the substance composing the membrane was poorly preserved. The peripheral ground-plasm was precipitated as a homogeneous or very finely granular region, distinct from the central ground-plasm. Again the comparative shrinkage effects could be noted by comparing the amount of separation of peripheral and central ground-plasms. With fixation in hot absolute, the amount of shrinkage was less than with cold absolute. It was very great with 70 per cent alcohol. In 30 per cent, however, the shrinkage was reduced to a minimum. The central and peripheral regions were in contact along the whole periphery (cf. Figs. 44, 45). The substance of the peripheral ground-plasm was in finer particles in the slides fixed with 30 per cent than with absolute alcohol. The peripheral ground-plasm was acidophilic but did not resist staining with basic dyes. The peripheral granules were found in a comparatively natural condition in nuclei fixed with absolute alcohol, and exposed to little contact with water, but were dissolved away or rendered much less conspicuous by washing (cf. Figs. 44 and 45). Reduction of the percentage of alcohol reduced the resistance of the granules to solution, so that after fixation in 30 per cent alcohol they were usually dissolved or modified even when contact with water was slight. The peripheral spherules were found after fixation in all grades of alcohol. They were usually regular in shape, but occasionally irregularities in outline and signs of fusion were observed (Fig. 47). The irregularities were never great, and were not increased by exposure to water. In many fixed nuclei the spherules were clumped near one pole and filled the whole peripheral region at that point (Fig. 43). During kinetophase they were found between the two poles, and often showed a tendency to form irregular rows at that stage (Fig. 47). It was interesting to observe that fixation in 30 per cent alcohol made the spherules more resistant to washing than fixation in absolute. This was thought at first to indicate solution of the spherule substance in alcohol, but after several days in absolute alcohol, no modifications beyond that of normal fixation were observed. In all cases the spherules were resistant to a high degree to staining with acid and basic dyes. In rare cases—and these usually near a division—the spherules were tinted very lightly by orange G and light green. They were never tinted by eosin. The endosomes were precipitated by all grades of alcohol, but the higher grades gave a more perfect picture of them. They were invariably stained with basic dyes, but never with the Feulgen reaction. After washing they were sometimes partially fused together (Figs. 45, 46) and always showed clear evidences of ragged and tattered edges. This effect was much more clearly indicated in elongated than in spherical endosomes. Lying between the chromosomes at the poles were the endosomal spherules in

dividing nuclei. These were usually invisible in nuclei fixed in low grades of alcohol, and very imperfectly preserved in higher grades (Fig. 47). There was no difference in their resistance to washing and their general staining reactions between hot and cold absolute fixation. The central region appeared as a reticulum or as a homogeneous region (Figs. 44, 45). In unwashed nuclei a very irregular and indistinct region at one pole of the central region was occasionally found. It had a higher affinity for basic dyes than the remainder of the central region, and it is thought that it represented a remnant of the hyaline body. It was never observed in washed nuclei. The ground-plasm itself showed no particular differences between fixation in low and high grades of alcohol, except that the particles appeared to be slightly smaller in nuclei fixed in hot absolute and 30 per cent alcohols. Shrinkage of the central ground-plasm appeared to parallel that of the peripheral ground-plasm, being lowest in hot absolute and 30 per cent alcohol. The ground-plasm was almost neutral in staining reactions, showing affinities for neither acidic nor basic dyes, nor resistance to staining with them. On occasions the central region was eccentric, suggesting the condition mentioned by Baker (1933). In nuclei in early kinetophase, strands appeared which seem to be identical with the chromosomal strands observed in compound fixatives (see below). In middle kinetophase, the chromosomes were found clumped together at the poles (Figs. 47, 48). In all cases they were greatly fused together, with indistinct outlines, showing clearly that they had been partially dissolved. This was most clearly observed in nuclei which had undergone prolonged washing. Lower grades of alcohol preserved the chromosomal substance less perfectly than higher grades. The chromosomes showed little affinity for basic dyes, and were but very slightly colored by the Feulgen reaction, or, more frequently, and always in the washed preparations, were completely negative to the Feulgen test. The chromosomal substance was colored rather deeply by light green and orange G.

After prolonged washing nuclei fixed with alcohol were sometimes entirely empty of all the nuclear elements. Only the nuclear membrane, sometimes retaining its spherical shape, and at other times greatly wrinkled and misshapen, remained. This occurred after all grades of alcohol, and, it may be noted here, after all other fixatives. It appears that this is due either to some abnormal condition of the nucleus itself or to uneven fixation. The fact that it occurred in all fixatives suggests that probably some abnormal condition of the nucleus itself occasioned the solution of all of the inner elements.

The results of alcohol fixation may be summarized in the following terms. Nuclear shrinkage is greatest in the medium grade of alcohol. The substance of the nuclear membrane is well preserved and resistant

to washing. The substance of the peripheral ground-plasm is most satisfactorily preserved in high and low grades and is resistant to washing. The peripheral spherules are well preserved and resistant to washing. The peripheral granules are imperfectly preserved, best by the highest grades, and are more or less soluble in water. The endosomal substance is imperfectly preserved, especially in lower grades, and especially in nuclei which are dividing or are in the early interphase stages. It is less soluble than the substance composing the peripheral granules, but shows distinct signs of dissolving, even in nuclei fixed in hot absolute alcohol. The central ground-plasm is preserved rather well in interphase nuclei, and is not dissolved during after-treatments. The chromosomes are very imperfectly preserved, especially in the lower grades of alcohol, and are clumped and partially dissolved during after-treatments even when fixed in absolute alcohol and exposed as little as possible to water. They are but slightly positive or wholly negative to the Feulgen nucleal test. The hyaline body, although well preserved in nuclei still lying in the fixative, is extremely soluble, and usually disappears almost entirely even when the contact with water is reduced to a minimum. On the whole, fixation appears to be very good while the nucleus still lies in the fixative, but during after-treatments aberrations of various kinds occur, which frequently produce extremely imperfect and unnatural images. Surprisingly enough the best fixation was obtained with the highest and lowest grades of alcohol, and the poorest by the medium grade. No explanation of this has been adduced.

Dioxane.—The use of dioxane for cytological technique is so recent that little is known of its effects on tissues and cells. It has replaced alcohol for dehydration to no small extent, especially before imbedding in paraffin, and since it was used for dehydration during part of this study, its effects on the nucleus were studied in order to determine if it was likely to affect the solubility of compounds precipitated by other fixatives.

The writer is not aware of any information concerning the chemical compounds precipitated by dioxane, nor their solubility after such precipitation. It is recommended by some cytologists to replace alcohol in certain of the alcoholic fixatives, and new formulae using dioxane as a fixative have been devised (see McClung, 1938).

In observations of the immediate effects of fixation, the death of the organism was instantaneous, and the nuclear changes also were instantaneous, resembling other fixatives more than alcohol in this particular. Cytoplasmic shrinkage was very small, but nuclear shrinkage was quite noticeable, and was estimated at about 20 per cent. The nucleus was drawn out of shape in many cases, usually becoming oval or indented

during the shrinking process. The nuclear membrane was poorly preserved. It was noticeably thinner, and in many cases the material composing the membrane ran into the cytoplasm in the form of irregular, short filaments of clear, hyaline material (Fig. 36). A profound alteration of the peripheral zone accompanied fixation. The ground-plasm was no longer distinctly differentiated from the spherules and granules. The spherules were partially dissolved and ran together confusedly, resulting in a very characteristic appearance. This may have resulted partially from a loss in distinctness of outline resulting from a change in refractivity, and results obtained in permanent preparations seemed to indicate that this was the case. The peripheral granules were apparently precipitated, but were little, if any, more prominent than in the living nuclei, and the indistinct nature of the background made it extremely difficult to observe them. The endosomes were distinct, regular in outline, and appeared to be well preserved. They were much more prominent in fixed than in the living nuclei. The central region contained a coarse precipitate in which neither hyaline body nor centriole was ever observed.

In permanent preparations the nucleus was deformed and had undergone a great deal of shrinkage. The homogeneous nuclear membrane showed a slight affinity for basic dyes, especially for haematoxylin. The peripheral spherules were present in large numbers in almost all nuclei, although in a few, rare cases they had been completely dissolved. Except for these exceptional nuclei, they appeared to be well preserved. They were resistant to staining with dyes, but occasionally were very lightly tinted by light green or orange G. The spherules were found in the median part of elongated dividing nuclei, where they were arranged in irregular rows and could be distinguished from the endosomal spherules by their staining reactions. The peripheral granules were present in nuclei which were exposed to very little water, but dissolved out very readily. Two hours of washing was sufficient to completely dissolve the peripheral granules. When present, they were basophilic. The endosomes were poorly preserved, usually being quite misshapen. They were distinctly less basophilic after washing, and in washed nuclei showed evidences of fusion and partial solution. In dividing nuclei the endosomal spherules were quite indistinct and frequently had lost their affinity for basic dyes to a considerable extent. Like the endosomes, they were often partially fused. The central region was not drawn away from the peripheral region, indicating very little shrinkage. It was somewhat more basophilic than the peripheral ground-plasm in unwashed nuclei, but this distinction was lost in washed nuclei. In unwashed nuclei the central ground-plasm showed a light, diffusely positive reaction to the Feulgen reagents during early and late kinetophase stages. In no case

was a hyaline body or centriole observed. Chromosomes were never found, although in several dividing nuclei a homogeneous region of differentiated material which, from its position, was apparently derived from the chromosomes was found. In unwashed material this region was somewhat more basophilic than the surrounding material.

The effects of dioxane may be summarized as follows. The process of fixation entails a considerable nuclear shrinkage, and appears to involve a poor preservation of the substance of the nuclear membrane. The apparent partial solution of the peripheral spherules is not observed in nuclei in permanent preparations, so it appears that this, and other observations of nuclei lying in the fixative, are partially determined by alterations in the refractive index resulting from the dioxane. The endosomes are apparently well preserved in nuclei still lying in the fixative, but show signs of solubility by fusion and loss of basophilic material in permanent preparations. The central ground-plasm is well preserved, insofar as shrinkage is concerned, but the basophilic elements are lost during extended periods of washing. The chromosomes are very poorly preserved, and the hyaline body and centriole seem to be completely lost, even in unwashed nuclei. Comparing this with the results obtained with alcohol, it is evident that the two reagents cannot be considered as identical in their effects. Neither reagent is sufficient, alone, to give good fixation, but they fail for different reasons. It is worthy of note that of all the reagents tried, dioxane was the only one to fix the nuclear membrane poorly, and there is some evidence that the substance of the nuclear membrane is soluble in dioxane.

Acetic acid.—Acetic acid is widely used as a nuclear fixative. It is not considered a satisfactory fixative alone, for it does not fix the cytoplasm and its effect on the chromatin is too violent. Baker (1933, p. 36) states, "Acetic acid is an excellent fixative for showing up nuclei clearly for histological work, but for a cytological study of the interphase nucleus it is useless. Chromosomes, however, it preserves excellently, and acetic acid is a component of all fixatives for chromosome studies. . . . Nevertheless it is not proper to use acetic acid (except perhaps very dilute) in studying the condensation of the chromatin to form chromosomes, nor the telophase transformation of the chromosomes into the interkinetic nucleus." Very little information is available concerning the chemistry involved in acetic fixation. Baker explains that its swelling action depends on the absorption of water because of an increase in the osmotic pressure in the fixed cell. The osmotic change is the result of the formation of a salt of the protein and the anion, which dissociates as a non-diffusible protein cation and an acetate anion, which is held to the cation by electrostatic attraction. The precipitate formed by acetic acid and albumin is

soluble in an excess of acetic acid. Nucleic acids, nucleoproteins, and nucleins are precipitated, the first two, at least, being insoluble in water. Acetic acid was used as a fixative at room temperature and warmed to 45-50° C. in this study. Glacial acetic, 10 per cent acetic, and 5 per cent acetic were used at both temperatures. Only room temperature was tried for solutions of 2 per cent, 1 per cent, and 0.5 per cent acetic.

Death was instantaneous with the acetic acid solutions used. A coarse precipitate of the cytoplasm formed immediately in all cases except fixation with 1 per cent and 0.5 per cent acetic. In these last mentioned dilutions the cytoplasm did not appear to be precipitated, and would disintegrate shortly after fixation, usually floating away and carrying the nucleus with it. For this reason, the observations of the nuclear effects with 1 per cent and 0.5 per cent acetic are less complete than in the case of the more concentrated solutions. Nuclear and cytoplasmic swelling occurred with fixation. The nuclear swelling was greater than the cytoplasmic swelling, reaching a maximum of about 76 per cent with glacial acetic. Even the lowest grades of acetic caused some swelling.

As a result of fixation, the nuclear membrane became much thinner. This appeared to be at least partially the result of the great swelling of the nucleus. It was homogeneous and somewhat less refractive than in life. The peripheral ground-plasm was precipitated as a finely granular region, with a number of prominent peripheral granules imbedded in it. The spherules were dissolved rapidly in the lower grades of acetic acid, but in the higher grades dissolved very slowly, or not at all. The endosomes were usually more prominent than in the living nucleus after fixation, except in the case of glacial acetic, when they were very little more refractive than in life. Usually homogeneous, the endosomes occasionally showed evidences of vacuolation. The central region was partially obscured by fixation, but appeared to be precipitated in very fine particles. Neither centriole nor hyaline body was ever observed in it. Acetocarmine and methyl green stained the peripheral granules and the endosomes, but not very selectively. All of the nucleus would gradually become stained, and frequently the cytoplasm, too, would stain with the basic dye. The first to stain, however, were the peripheral granules and endosomes. No evidence of a distinct basophilic nature could be detected in the central region.

In permanent preparations the nuclear membrane was homogeneous, staining quite deeply with the acid dye. After fixation in hot acetic acid, it showed a slightly higher affinity for basic dyes than in the other cases. In the lower grades of acetic, the nuclear membrane appeared to be thinner than in the higher grades (cf. Figs. 49, 53). Nuclei fixed in grades lower than 5 per cent frequently appeared greatly wrinkled, ap-

parently a result of incomplete fixation of the material within the nucleus as well as of the nuclear membrane.

The appearance of the peripheral region depended to a great extent on the grade of acetic acid used. The ground-plasm was homogeneous in nuclei fixed in glacial acetic acid, sometimes indistinguishable from the central ground-plasm, and sometimes clearly separate (cf. Figs. 51 and 53). With increased contact with water the differentiation of the central and peripheral ground-plasms decreased, but this may have been partially an effect of the destruction of the endosomes and the peripheral granules during washing. In 10 per cent acetic the peripheral ground-plasm was usually an irregular reticulum. In 5 per cent acetic, the peripheral ground-plasm was more or less dispersed if fixation was carried out at 45° C. At room temperature with 5 per cent acetic and all of the lower grades, there was no peripheral ground-plasm, as such, in the nucleus. All of the nuclear contents were clumped together in the center or at one side of the nucleus. This clumping of substances was probably at least partially caused by the absence of a supporting peripheral ground-plasm. It seems, from the differences between fixation of the peripheral ground-plasm in hot and cool 5 per cent acetic acid that the precipitation of the peripheral ground-plasm in the hot 5 per cent acetic was due to the heat and not the action of the acetic. The peripheral spherules were partially destroyed in hot glacial acetic acid (Fig. 51), and were increasingly dissolved as the percentage of acetic acid was reduced. They were more satisfactorily preserved in nuclei fixed with hot acetic than cold acetic. Even with the warmed fixative, they were completely destroyed in nuclei fixed with lower grades of acetic. The peripheral granules were most prominent in nuclei fixed with 10 per cent acetic (Fig. 49). In higher and lower grades they were rarely found, except in occasional nuclei in the unwashed preparations (Fig. 53). In washed slides in all percentages of acetic acid, used either hot or cold, the granules were dissolved (Fig. 52).

The endosomes were poorly preserved by acetic acid in most cases. With glacial acetic fixation they are quite basophilic, but tend to fuse together, especially after washing (Figs. 52, 53). When the endosomes have not fused together the differentiation of a darkly-staining cortex and lightly-staining core is commonly observed (Fig. 51). Not uncommonly the endosomes are completely dissolved in preparations washed for prolonged periods. Insofar as the endosomes were concerned fixation in 10 per cent acetic was like the glacial acetic fixation. The results of cold 10 per cent acetic fixation were similar to those observed in hot 5 per cent, and in cold 5 per cent acetic and all lower grades, the endosomes were quite soluble. The tendency toward fusion of the

endosomes in nuclei fixed in 10 per cent acetic acid was less marked than in nuclei fixed with glacial acetic, especially when the nuclei were in the early interphase condition. Hot fixation usually favored endosomal fusion. The observations of endosomal spherules closely paralleled those on the endosomes, whenever they were observed. In the glacial acetic preparations no dividing nuclei were found, so that the observations are incomplete. Similarly none were found in the lowest grades, and all observations are on dividing nuclei which were encountered in slides fixed in either 10 per cent or 5 per cent acetic.

The central region appeared to be homogeneous after fixation in glacial acetic, and usually merged with the peripheral zone. In some cases the central region was more basophilic than the peripheral zone (Fig. 51), and in early or late kinetophase a diffusely positive Feulgen reaction was observed, even in washed nuclei. Although the central region was homogeneous after fixation in hot 10 per cent acetic, the cold 10 per cent fixation gave it a granular appearance (Fig. 49). With hot 5 per cent acetic the central region was fibrillar and granular, and several nuclei in which remnants of a hyaline body could be seen were found. Fixation in cold 5 per cent, or in lower grades was so incomplete that no observations could be made successfully. So much of the nuclear substance was dissolved that identification of particular elements was uncertain. In several nuclei fixed with glacial acetic, remnants of the centriole were found, and one nucleus seemed to contain a dividing centriole (Fig. 53). Chromosomes were well preserved by acetic acid, especially in 10 per cent. Their contours were regular, and appeared quite precise. The staining reaction with Feulgen appeared to be positive, although so few dividing nuclei were present in the Feulgen material that a definite statement cannot be made. With haematoxylin, the chromosomes showed a slight affinity for the basic dye.

Summarizing the results of the observations on acetic acid fixation, the following conclusions may be drawn. Acetic fixation involves a considerable swelling of the nucleus and an accompanying attenuation of the nuclear membrane. The substance of the membrane is usually well preserved, except in low grades of acetic. The peripheral ground-plasm is precipitated in an insoluble form by the high grades of acetic, but in the lower grades the solubility of the substances precipitated increases. Peripheral granules are never well preserved, but are found most satisfactorily preserved in the highest grades of acetic only. The peripheral granules are preserved most satisfactorily by 10 per cent acetic acid, and with an increase or decrease are usually absent, or are less basophilic. Endosomes are poorly preserved by acetic acid, usually fusing together, or dissolving completely during staining procedures. Immediately after

fixation, however, before exposed to water for any length of time, they appear to be comparatively well preserved. The central region contains a centriole in some nuclei, although this is not invariably true. The hyaline body is not preserved. The ground-plasm is precipitated with little shrinkage in high grades of acetic, but as the percentage of acetic acid decreases, the fixation is progressively poorer, the precipitate formed becoming more soluble in water. Chromosomes are well preserved, especially in 10 per cent acetic, and a positive reaction with the Feulgen test is suggested.

Mercuric chloride.—A saturated solution (about 6.5 to 7 per cent) of mercuric chloride was used for fixation at room temperature and at 45-50° C. A 3 per cent solution was also employed at room temperature. The detailed effects of fixation were somewhat different in the three procedures. In the study of the immediate effects of fixation, a saturated solution was used.

The action of mercuric chloride during fixation is not very completely understood. Mann (1902, p. 77), writes, "With ammonia it [mercuric chloride] forms the mercuriammonium chloride or infusible white precipitate HgCl_2 plus $2 \text{NH}_3 = \text{NHgH}_2\text{Cl}$ plus NH_4Cl . Analogously to this equation it probably combines with the nitrogenous constituents of the cell." This long-accepted view of sublimate action was denied by the work of Thomas and Norris (1925) who found that the coagulation of albumin produced by ferric chloride in dilute solutions was soluble in an excess of ferric chloride, but that a new precipitate appeared in more concentrated solutions. Baker (1933) believes that sublimate acts in the same way as ferric chloride. The precipitate formed in dilute solutions is a compound, a mercury or ferric albuminate, during the formation of which the albumin is acting as an anion because the salt is on the basic side of the isoelectric point. But as the concentration is increased the pH of the fluid gradually goes toward the acid side due to the ionization of the mercuric chloride to hydrogen and chloride ions plus $(\text{HgCl})_2\text{O}$ or HgClOH . Mann's statement that it ionizes to HgOH and 2HCl was disproved by Luther (1904). As the concentration of the fixative is increased a precipitate of denatured albumin forms for entirely different reasons than the first. This denatured protein, different from the denatured protein resulting from precipitation by alcohol, carries a varying amount of the mercury salt with it. No union between these occurs, however, for all the mercury can be washed out. Thomas and Norris consider that this precipitation with ferric chloride is identical with heat coagulation. This view depends on the fact that salts are invariably necessary for heat coagulation and that salts of heavy metals are the only ones which will cause precipitation of proteins at room temperatures.

It is worthy of note that the heat coagulations of albumins in general has been known to require hydrogen ions for a long while. Mann (1902, p. 67) says, "Any factor which tends to prevent the formation of hydrogen ions will also prevent coagulations." Any salt of a heavy metal favors the liberation of hydrogen ions. This may be an important factor in producing the coagulation resulting from mercuric chloride solutions.

Fischer (1899, p. 23) reports, "Wie diese [Peptone, Deuteralbumose, and Protalbumose], werden auch Albumin, Globulin, Casein, und Conglutin, Hämoglobin, Nuclein und Nucleinsäure als alkalischer und säurer Lösung unlöslich ausgefällt."

Not much of importance has been found concerning the desirability of mercuric chloride as a fixative. Strangeways and Canti (1927) report that a coarse precipitate is formed in nucleus and cytoplasm by this salt. Baker (1933) accuses it of causing a very unlikelike fixation, and says that the good stainability of material after sublimate fixation is its chief advantage.

Death in mercuric chloride is instantaneous, although several moments are required for fixation to go to completion. Cytoplasmic shrinkage is considerable, but nuclear shrinkage is small. The nuclear membrane was homogeneous after fixation, but a distinct loss in refractivity was observed. In the peripheral zone the ground-plasm was precipitated in small particles. The whole of the ground-plasm appeared to be homogeneous after fixation. Peripheral granules were not visibly altered during fixation, and did not increase in refractivity or numbers. The peripheral spherules were not visibly altered when the sublimate first came in contact with the nucleus, but in about a minute they became noticeably smaller, and were usually completely dissolved in about two minutes. No visible changes other than a gradual decrease in size accompanied their solution. The endosomes appear to be homogeneous after fixation, and much more refractive than in life. The central region was almost homogeneous after fixation in some nuclei, and was finely granular in other nuclei. In a few cases a central granule could be observed in the center of the central region. In nuclei in the correct stages a hyaline body could be observed. It was indistinguishable from the endosomes insofar as shape or refractivity were concerned, but occupied its characteristic position, and was stained much more lightly with methyl green than the endosomes.

In permanent preparations the nuclear membrane was homogeneous and distinctly acidophilic. It was consistently thinner than in permanent preparations fixed with compound fixatives, but there was no indication of solution of the substance composing it even after prolonged washing.

The peripheral ground-plasm was a homogeneous region, composed of

fine granules. It was acidophilic, although in a few nuclei it was tinted lightly by the basic dye, especially in nuclei fixed with the cold saturated solution. The peripheral spherules were excellently fixed by the hot saturated mercuric chloride, almost filling the whole peripheral region, and obscuring any peripheral granules which might have been present. Peripheral spherules were but very occasionally found in nuclei fixed in the cold saturated, and never in the 3 per cent, solutions. Hot and cold saturated solutions caused a slight separation of the central and peripheral regions, but this effect was little noticed in nuclei fixed with 3 per cent sublimate. Peripheral granules were occasionally found in the nuclei fixed with a cold saturated solution, but were much less prominent in material fixed in a 3 per cent solution.

The endosomes were essentially the same in all of the sublimate-fixed nuclei. They were distinctly basophilic, but remained uncolored by the Feulgen reaction. The larger spherical endosomes were quite frequently vacuolated, but the elongated endosomes never showed this structure. The endosomes were always sharply outlined, and the substance, except for the vacuolated ones, was homogeneous throughout.

The central region was finely granular and reticular in saturated solutions, and homogeneous in 3 per cent solution. In nuclei fixed in 3 per cent sublimate, the differentiation of the peripheral and central ground-plasms was very difficult, for they were not drawn away from each other, and both were homogeneous and acidophilic to very slightly basophilic. After fixation in cold sublimate only was a hyaline body observed. It was most prominent in Feulgen-light green preparations, where it was distinguished by its affinity for light green and its clear hyaline appearance.

Two interesting points appear in the results of mercuric chloride fixation. The first is the differences observed in the size of the particles of the precipitated ground-plasm after treatment with dilute and saturated solutions of sublimate. The fine particles produced during fixation with the 3 per cent solutions cause a homogeneous appearance which contrasts very strongly with the finely granular appearance resulting from fixation with saturated solutions. When albumin is precipitated in test-tube experiments, active precipitants have a tendency to form flocculent precipitates, while less active ones show a light opalescent coloration. These opalescent colors in the test-tube experiments appear to be comparable to the homogeneous appearance resulting from fixation in the dilute solution. The coarser precipitate forms as a result of stronger concentrations in both nucleus and cytoplasm. If the coagulation of albumins by mercuric chloride is comparable to heat coagulation, an increased effect should be apparent in the heated solutions. No such sum-

mation effect was found, except for the preservation of the peripheral spherules, which were preserved only in the heated solution. In other simple fixatives the heated solutions frequently showed a more accurate and complete preservation of the peripheral spherules whether the cold fixative did or did not preserve them, which seems to indicate that the peripheral spherules are preserved by heat. The peripheral spherules, however, are soluble in cold mercuric chloride, as was observed in studies of the immediate effects of fixation. A greater amount of detailed work is necessary before any conclusions can be drawn, but the inference seems clear that, regardless of the way that albumins are precipitated by mercuric chloride, the mercuric chloride fixation of the spherules does not resemble heat coagulation. It appears that in the case of many fixatives their total effect is very incompletely known, and they may react very differently with different elements.

Another interesting observation was made on mercuric chloride fixation. The nuclei were extremely lifelike in appearance, particularly after fixation in a hot saturated solution. This appears to be different than is the case with most other types of nuclei, which are said to be abominably fixed by sublimate. In the case of *E. blattae*, the nucleus is extremely lifelike, marred only by the very finely granular appearance of the peripheral ground-plasm, and the slight increase in refractivity of the central ground-plasm. Even shrinkage is extremely small.

Formaldehyde.—Undiluted formalin (40 per cent formaldehyde) was used for fixation at room temperature and at 45-50° C. A 10 per cent solution of formalin (4 per cent formaldehyde) was used at room temperature.

Most of the observations on fixation processes were made with undiluted formalin. A few trials with the 10 per cent solution were undertaken, but the results of the first few coincided with the observations made with formalin, and no great number were attempted. Not enough work was done with the 10 per cent solution to draw any valid conclusions.

Cytoplasmic shrinkage was very small, and the nuclear shrinkage was so slight that none could be measured. Death was instantaneous and all of the visible changes accompanying fixation were completed in one minute. At the moment of fixation the nuclear membrane appeared to become somewhat thinner. The substance of the membrane remained homogeneous, and no alteration of refractivity was observed. The peripheral zone underwent very little visible alteration during fixation. The peripheral ground-plasm remained homogeneous, and no precipitate was formed. The peripheral spherules became slightly smaller, and appeared to be a little less refractive, but no other changes could be observed. The peripheral granules were no more distinct after fixation than before

fixation. The endosomes gradually became more prominent, until about a minute after the fixative was applied. They appeared to be homogeneous, and vacuolation was not observed. The central region remained hyaline after fixation. No visible precipitation occurred, and the central ground-plasm remained invisible.

In permanent preparations the nuclear membrane appeared to be homogeneous and acidophilic. It was usually most deeply stained with light green. In some cases where shrinkage had occurred during dehydration the membrane was greatly wrinkled. This was most noticeable in sectioned amoebae which had been subjected to prolonged periods of washing in running water. In unwashed preparations this was very rarely noticed. There were no obvious visible differences in the fixation with undiluted and 10 per cent formalin.

The peripheral zone was very characteristic in appearance after formalin fixation. The ground-plasm was homogeneous and showed some affinity for both acid and basic dyes. It was difficult to observe because of the large numbers of peripheral spherules which were invariably present (Fig. 54). Hot and cold fixatives appeared to preserve the spherules equally well, and they showed no tendency to dissolve after fixation in the 10 per cent solution. They were invariably resistant to staining with both acid and basic dyes, but were occasionally very lightly tinted with the acid dyes. No evidences of fusion or partial disintegration were ever observed. The peripheral spherules were present in such large numbers that they usually obscured the peripheral granules, just as they do in living nuclei. In some cases a few granules, rather basophilic, could be found.

In all preparations the endosomes were homogeneous unless very large, when vacuoles were occasionally observed. They were stained with basic dyes, but were also deeply colored by acid dyes in wholly destained preparations and in Feulgen-light green preparations. They were not colored by the Feulgen reaction. The basic dyes appeared to stain the endosomes less deeply than after some of the other fixatives. Occasionally a dark basophilic granule was observed in the center of larger, non-vacuolated endosomes. No alteration of the endosomes resulted from prolonged washing.

The central region was homogeneous, and indistinguishable from the peripheral ground-plasm. In no case were the central and peripheral ground-plasms separated by fixation shrinkage, either in washed or unwashed preparations (Fig. 54). In most nuclei the central ground-plasm was stained very lightly with the acid dye, or occasionally, very lightly with the basic dye. Centriole chromosomal strands and hyaline bodies were sought in vain. During division the central region changes

could not be studied, and the chromosomes were not distinct (Fig. 55), but formed a homogeneous region at the pole of the dividing nuclei, slightly tinted with purple in Feulgen preparations. These nuclei were almost identical in appearance with the descriptions given by Kudo (1926) of living nuclei during division, except that the clump of material at the pole of the nuclei which was not invaded by the peripheral granules and spherules was tinted with the basic dye or with the Feulgen reaction.

It was interesting to observe that nuclei parasitized by *Nucleophaga*, described by Mercier (1907, 1910) and Kirby (1927), appeared in nuclei fixed with formalin in an extremely lifelike condition (Fig. 56). These nuclei were characterized by the lack, or reduced amount, of endosomal substance, and the presence of the large spherical parasites.

As can be seen from the above description, the action of formaldehyde leaves the nucleus in a most lifelike condition, insofar as appearance is concerned. This is especially unexpected when one considers the large number of chemical reactions which supposedly accompany fixation with formalin (see Mann, 1902). Fixation of most of the substances entails the formation of additive compounds with formaldehyde, with the release of water. The structures are apparently coagulated without causing any great alteration of the physical state of the nucleus, resulting in a very slight alteration of the refractivity of the parts. This is not a new observation, for Noël and Mangenot (1922), after using formaldehyde on a large number of different kinds of plant and animal nuclei, came to the conclusion that formalin, diluted, is an excellent fixative. Baker (1933, p. 32), remarks, "Noël and Mangenot (1922) claim that the use of formaldehyde gives a very lifelike fixation of the nucleus, unlike standard fixatives. For some reason it is an atrocious fixative for the mammalian testis. Shrinkage is so great that sections are scarcely recognizable." Fixation of *E. blattae* appears to resemble that reported by Noël and Mangenot, especially for organisms fixed by the smear technique. In sectioned preparations, especially if any prolonged period of washing is allowed, considerable shrinkage occurs.

Indeed, formalin fixation is so lifelike that it is hardly more advantageous for study than the living nucleus, except for the clearer image of the endosomes made possible by staining, and the permanence of the preparations. In only one respect does *E. blattae* differ from the material studied by Noël and Mangenot. They report that chromosomes are excellently preserved, while in *E. blattae* they are not distinct.

Chromic acid.—The study of chromic acid fixation was made with a one per cent solution, used at room temperature and at 45-50° C. for permanent slides, and at room temperature for the observation of the immediate effects of fixation.

Death was instantaneous in chromic acid, but the visible alteration of the nuclear elements was not complete for several minutes. Cytoplasmic and nuclear shrinkage was very slight. The nuclear membrane became somewhat thinner at the moment of fixation, but remained homogeneous and highly refractive. The peripheral ground-plasm was precipitated in fine granules which clumped together in indefinite aggregations and usually obscured the peripheral granules to some extent. The granules, insofar as they could be observed, underwent no visible changes, and were not more refractive after fixation. At the moment of fixation the peripheral spherules became darker and noticeably less refractive. In a few moments they began to dissolve, and gradually disappeared. There was no evidence of fusion of the spherules while they dissolved. The endosomes were well preserved. They were distinctly outlined, and were composed of two types of substances which were visibly differentiated. The outer layer of the endosomes was more dense and refractive. The inner portion was composed of material apparently less dense and less refractive. The larger endosomes showed a vacuolated structure with the less refractive material collected in the vacuoles. The central ground-plasm was precipitated in fine particles which tended to aggregate into clumps similar to those observed in the peripheral ground-plasm. The hyaline body and centriole were never observed.

In permanent mounts made with material fixed in chromic acid, the nuclear membrane retained its homogeneous and refractive nature. The membrane was invariably acidophilic, although in a few cases some affinity for the basic dyes was also noticed. The peripheral ground-plasm was precipitated as a coarsely granular region which usually contained a combination of the acid and basic dyes (Fig. 58). Scattered throughout the peripheral region were the basophilic peripheral granules. No indications of solution of the peripheral granules were noticed in the slides washed for prolonged periods. The peripheral spherules were not found, even in slides in which contact with water had been reduced to a minimum. The endosomes usually appeared to consist of an outer, more basophilic cortex, and an inner, less basophilic core. The outer material was sharply outlined, and distinctly differentiated from the inner material. In some cases the endosomes were vacuolated. There was no evidence of solution of the material composing the endosomes in the washed slides. The endosomes were not colored by the Feulgen reaction. The central ground-plasm formed a coarse precipitate which was not visibly differentiated from the peripheral ground-plasm, and reacted similarly to it with basic and acidic dyes. Nuclei in which the chromosomal anlage were visible, however, showed a distinctive differentiation of central and peripheral regions, with a more homogeneous appearance of the central region and slightly basophilic strands scattered throughout the central

region (Fig. 57). The hyaline body was never observed. The centriole, likewise, was not visible. No dividing nuclei were found in the preparations made with chromic acid.

Although chromic acid has been used on animal substances in tanning for many years its action is not completely understood. When it strikes the cell, denaturation of the protein is thought to be the first step in fixation. A slower hardening action is also involved, according to Berg, who attributes this to the anion HCrO_4 . The fixation process seems to be accompanied by the formation of compounds of the protein with the chromic acid, but the manner of its formation is not very clearly understood. Berg (1927) believes that this compound is linked with the oxidizing powers of the acid. Baker (1933, p. 42) says, "Exactly what happens is not known, but part of the process is an oxidation, resulting in the formation of Cr_2O_3 , which gives a greenish color to the tissue. This is certainly not all that happens, for oxidizing agents are by no means necessarily fixatives." Its action may be summarized by saying that it is a precipitant for almost all proteins. It is thought by Baker to have no effect on the fat or mitochondrial substance. In view of the incomplete knowledge of the effects of chromic fixation it is impossible to draw any immediate conclusions from the observed morphological changes in the cell which it causes.

Picric acid.—Picric acid fixation for permanent mounts was done with a saturated solution used at room temperature and at 45-50° C. Observations on the immediate effects of fixation were made with a saturated solution used at room temperature.

Fixation with picric acid was very rapid, occurring in less than a minute, insofar as visible changes are concerned. Death was instantaneous, and cytoplasmic shrinkage was very great. Nuclear shrinkage was somewhat smaller than cytoplasmic shrinkage. The nuclear membrane was homogeneous after fixation. It did not undergo shrinkage, and remained homogeneous and refractive. The peripheral ground-plasm was formed as a rather coarse yellow precipitate. The spherules disappeared during fixation, and the granules were usually obscured by the precipitated ground-plasm. The endosomes were homogeneous, and appeared to be well preserved. The central ground-plasm was precipitated as a coarse material, usually drawn away from the peripheral region rather considerably. This tendency towards separation of the central and peripheral regions, noticed in many other fixatives, was accompanied by an unusual condition. The endosomes, which usually remained at the surface of the peripheral region when the two were separated in other fixatives, consistently clung to the central region and were drawn away from the peripheral zone in picric acid.

In permanent preparations the nuclear membrane was homogeneous

and refractive, and invariably acidophilic. The membrane appeared to be thicker in nuclei fixed with picric acid than with other fixatives. The peripheral ground-plasm was drawn away from the central region, and appeared to be finely granular. Imperfectly preserved peripheral spherules were occasionally found imbedded in the ground-plasm, but they were never present in large numbers as in living nuclei, and usually were absent. Basophilic granules were present, staining darkly with haematoxylin and safranin. The endosomes were well preserved. They were homogeneous, distinctly outlined, and almost hyaline when completely decolorized. In many cases a more basophilic cortical substance was observed in the endosomes. The central ground-plasm was separated from the peripheral region, and appeared as an acidophilic, reticular region. A centriole was occasionally observed. The hyaline body was never prominent, but traces of that structure were occasionally found. When present it tended to be slightly acidophilic. Dividing forms were not numerous, so the structure of the dividing nuclei could not be studied very thoroughly. The chromosomes appeared to be rather poorly preserved, with indistinct outlines and evidences of fusion, particularly in the washed preparations. They were acidophilic, being rapidly decolorized. No dividing nuclei were found in the Feulgen preparations.

Picric acid precipitates proteins in the same way that other compounds containing complex anions act. The anion forms a chemical compound with the protein; in the case of picric acid, a protein picrate. Picric acid is said to have no effect on lipides, but to preserve albumin, globulin, nuclein, and nucleic acid. The precipitate of nucleic acid is soluble in water, according to Mann (1902). Jones (1920) says that picric acid precipitates only protein from the nuclein solutions, leaving the nucleic acid in solution. The failure to precipitate nucleic acid may very possibly be involved in the apparent poor preservation of the chromosomes by picric acid. Since the endosomes appear to be very well preserved, it seems to indicate that they are precipitated entirely, and not in protein and nucleic acid fractions. If they are precipitated in protein and nucleic acid fractions, the nucleic acid must dissolve very quickly, for no indications of a positive Feulgen reaction was obtained in the endosomes, even when the contact with water was reduced to a minimum.

Osmium.—Fixation for permanent mounts was accomplished with osmic vapor used for 45 seconds to one minute, and a 2 per cent solution of osmium tetroxide at room temperature. For the study of the immediate effects of fixation the 2 per cent solution was used at room temperature.

At the moment the fixative was applied the first group of visible alterations occurred instantaneously. There was no noticeable shrinkage

of cytoplasm or nucleus. The cytoplasm remained very lifelike, and the nucleus retained its normal size and shape. The nuclear membrane became noticeably darker and was less refractive. It gave an added indication of an alteration in its substance by a marked affinity for methyl green. It remained homogeneous, however. The peripheral ground-plasm remained homogeneous after fixation. For about ten minutes the spherules were visible, not altering their appearance in any way. After this they gradually disappeared, and finally were entirely dissolved. The peripheral granules became a little more prominent and refractive during fixation. For the final appearance of the nucleus to be attained (Fig. 37) required, on the average, about fifteen minutes. The central region was precipitated in a finely granular condition in all cases. A centriole was never observed, possibly because of the granular nature of the precipitated central ground-plasm. The hyaline body was distinct after fixation of nuclei in the late kinetophase or early interphase (Fig. 37). It was homogeneous and retained its characteristic position at one pole of the central region. Unlike its appearance in many other fixatives, especially when studied in permanent mounts, its outlines were regular and distinct. It had a slight affinity for the basic dyes. The endosomes were coagulated by osmium, usually appearing to be perfectly homogeneous, but in rare cases of large endosomes, showing a vacuolated inner structure. They were invariably distinctly basophilic, staining deeply with methyl green.

In permanent preparations the nuclear membrane remained homogeneous and basophilic. The peripheral ground-plasm was homogeneous, and stained lightly with basic dyes before differentiation. The basic dyes were rapidly extracted, and the ground-plasm stained darkly with acid dyes. Peripheral spherules were not found in nuclei fixed with osmium tetroxide solution, but occasionally could be found very imperfectly preserved and much misshapen in preparations fixed with the osmium vapor. The peripheral granules were invariably basophilic, but never so deeply basophilic as the endosomes. The endosomes were not very well preserved, and in many cases the outlines were rather indistinct, especially in nuclei fixed with osmium vapor. The remnants of such endosomes stained with the basic dye. In endosomes fixed with the solution, the shape was frequently well preserved, and the endosomes showed a distinct differentiation of an inner less basophilic material and an outer more basophilic material. The inner substance was not infrequently gathered in vacuoles in larger endosomes. The central region was well preserved. The ground-plasm was precipitated in fine particles which did not form a reticulum. In some nuclei the basophilic centriole could be seen. A hyaline body was not observed in most nuclei, but in some

cases it was visible in an incomplete form. Its reaction to stains varied somewhat in different nuclei, ranging from slightly basophilic to somewhat acidophilic.

Osmic fixation is thought to occur in two steps. The primary action, described by Berg (1927), involves a combination of the molecule with the protein amino-groups. The second action is associated with a gradual oxidation of the compound formed and is accompanied by a blackening of the tissues and a reduction in their stainability. Osmium preserves things in a very lifelike state, especially the fats and lipides. Baker (1933) reports that cells are more lifelike after osmium fixation than any other simple fixative. It was the more remarkable in view of these statements, to observe that the nucleus was not as lifelike after fixation with osmium as after formaldehyde. Cytoplasmic fixation was superior to that observed for any other simple fixative. Except for the refractive peripheral spherules and the central region, osmium fixation is rather lifelike, while the nucleus is still lying in the fixative. New unlikelike appearances creep in during the washing and staining procedures, causing the final appearance in permanent mounts to be quite different from that of the living amoeba.

B. COMPOUND FIXATIVES

As most of the changes in nuclear appearance resulting from fixation with the compound fixatives survived the washing, dehydration, and staining procedures involved in making permanent slides, only the observations made on nuclei during fixation are given below. A few detailed differences may be found in the discussion of nuclear morphology in the trophic nucleus in a later section. Many of the effects of fixation with compound fixatives showed rather clearly a summation of the effects of the reagents composing them.

A variety of compound fixatives was tried, but for most of the descriptive work, at least, six were used almost to the exclusion of the others. These were Gilson-Carnoy, Schaudinn, Carnoy, Bouin, Zenker and Flemming. A brief discussion of these will precede the observations of fixation.

Gilson-Carnoy, a powerful fixative composed of a saturated solution of mercuric chloride in six parts of absolute alcohol, three parts of chloroform, and one part of glacial acetic acid, is the most vigorous fixative used in this study. It is generally recognized as an extremely penetrating fixative, and is not recommended for cytological work on delicate structures. The chief objection to it given in most technique manuals is the shrinkage which it is reputed to cause. It is said to leave the nucleus and cytoplasm in a most unlikelike condition.

Schaudinn is composed of 2 parts of a saturated solution of mercuric chloride and one part of absolute alcohol, plus one to five per cent of glacial acetic acid. In this study the acetic acid was varied between one and two per cent. This rather strong and penetrating fixative is an active precipitant of almost all the substances found in the cytoplasm and nucleus. Although it is widely used among protozoologists it is not popular with cytologists. Baker (1933) does not even mention it. McClung (1938) recommends it as a standard routine fixative for protozoan work, but not to be used for special investigation without supplementation with other fixatives. Langeron (1925) similarly relegates its use primarily to protozoan work, but recommends either alcoholic or aqueous Bouin's as being equally satisfactory.

Carnoy is composed of 3 parts of absolute alcohol and one part of glacial acetic acid. It is a strong precipitant and penetrates rapidly. Baker (1933, p. 65) says, "The fluid is a rational one, for the acetic prevents the shrinkage and extreme hardening which would be caused by the alcohol. The alcohol fixes the cytoplasm and the acetic acid the nucleoproteins." According to Baker it dissolves lipides, and precipitates glycogen. It is a good fixative for chromosomes but cannot be used for Golgi and mitochondria. Most of the remainder of the handbooks on microscopical technique recommend Carnoy as a rather good fixative for nuclear detail if accompanied by fixatives of other types.

Aqueous Bouin, composed of 25 parts of 40 per cent formaldehyde, 5 parts of glacial acetic acid, and 75 parts of a saturated solution of picric acid has long been recognized as one of the most dependable of general fixatives. Baker (1933, pp. 65, 66) remarks, concerning the general properties of Bouin as a fixative, "Mitochondria are not usually fixed. The fluid penetrates quickly and fixes evenly. . . . The chromosomes are no doubt fixed mainly by the acetic and the formaldehyde probably restrains the too coarse precipitation of the cytoplasm by the picric and of the nucleoproteins by the acetic, while the picric gives a sufficiently soft consistency for easy sectioning and makes staining easy."

Zenker is not a standard cytological fixative. It is not mentioned by Baker (1933) and is but briefly mentioned in most other handbooks of cytological technique. It is composed of a solution of 2.5 per cent potassium bichromate, 1 per cent sodium sulphate, 5 per cent mercuric chloride, and 5 per cent acetic acid. It has not been used extensively in studies on the nucleus in Sarcodina, but has been highly recommended as a fixative for studies of the cytoplasmic fibrils and other structures in ciliates.

Flemming, composed of 15 parts of a 1 per cent solution of chromic acid, 4 parts of a 2 per cent solution of osmium tetroxide, and 1 part of

glacial acetic acid is one of the most widely used and best liked fixatives. Baker (1933) observes that it frequently does not fix evenly. This difficulty was encountered in colons preserved whole for sectioning, when the amoebae along the margin of the colon were much more satisfactorily preserved than those in the center. Baker explains its action in the following way (p. 68), "The osmium gives a homogeneous fixation of the cytoplasm, prevents too crude a precipitation of the nucleoproteins of the interkinetic nucleus, and preserves and blackens fats, while the acetic acid and chromic preserve the chromosomes admirably. The chromic further gives sufficiently firm consistency and facilitates the staining of chromosomes with the basic dyes." In preparations made for this study Flemming fixation was exceptionally good in most respects.

With all of these fixatives death was instantaneous. The effects of fixation on the various nuclear elements, with very few exceptions, reached completion so rapidly that each structure had to be studied separately and observed many times in order to gain a complete view of what happened during fixation. Visible alterations were usually complete after the first five to fifteen seconds, except in Zenker and Flemming, when some of the effects were quite deliberate.

The strong fixatives, Gilson-Carnoy, Schaudinn, and Carnoy, form a coarse cytoplasmic precipitate. Alveolation is somewhat less marked in Carnoy than in the other two strong fixatives. Cytoplasmic alveolation was still less developed in Bouin. A very coarse cytoplasmic precipitate was formed during fixation with Zenker, but alveolation was very slight. Cytoplasmic fixation was most nearly lifelike with Flemming, where a fine precipitate and almost no alveolation was the rule. Cytoplasmic shrinkage was very great with Gilson-Carnoy and Schaudinn. Carnoy and Bouin bring about much shrinkage, although less than the preceding two fixatives. Shrinkage in Flemming and Zenker is extremely small. It appears from these results that there is very little correlation between the size of the particles precipitated and the degree of shrinkage and alveolation. As might be anticipated, however, there is a rather direct correlation between the amount of cytoplasmic shrinkage and the degree of alveolation.

Nuclear shrinkage is not directly correlated with the cytoplasmic shrinkage. The greatest shrinkage of the nucleus was observed consistently in Gilson-Carnoy, Schaudinn, and Flemming. Carnoy caused marked shrinkage, but less than the above-mentioned fixatives. The smallest nuclear shrinkage was caused by Bouin and Zenker fixation. The large amount of nuclear shrinkage during Flemming fixation was surprising in view of its accepted excellence as a nuclear fixative. Although repeated many times, the same amount of shrinkage was invariably

found. In spite of this, the spatial relationship of the various nuclear elements remained unchanged, and accurate presentation of nuclear structure invariably resulted from Flemming fixation.

Visible alterations in the nuclear membrane were very slight during fixation. The advancing wave of fixative always elevated the cover slip slightly, so that the nucleus was less compressed and the membrane appeared somewhat thicker. Because of this it was impossible to estimate accurately the shrinkage or swelling of the nuclear membrane during fixation. The highly refractive membrane always appeared somewhat less refractive after fixation with Gilson-Carnoy, Schaudinn, and Flemming fixatives. Part of this loss in refractivity of the membrane undoubtedly came as a result of the increased refractivity of the cytoplasmic substances. However, the other fixatives used, which also caused a change in the refractivity of the cytoplasmic substances, left the nuclear membrane almost unchanged. For this reason it appears probable that there was some direct effect of the first three fixatives on the nuclear membrane. A definite precipitation of the substances composing the nuclear membrane was never observed. The membrane remained clear and hyaline in all cases. During the shrinkage of the nucleus it was hoped that some evidence of the hyaline cytoplasmic membrane investing the nucleus, described by Bütschli (1878), might be found. Careful observation failed to reveal any trace of this structure. The nuclear membrane remained homogeneous in all fixatives, and the bilamellar structure of the membrane described by Schubotz (1905) was never observed. Striations, possibly canals extending through the nuclear membrane, as described by Sassuchin (1930) were also looked for in vain. The nuclear membrane in the fixed state was invariably acidophilic, or, at least, showed no affinity for methyl green or acetocarmine beyond that showed by the cytoplasm, unless fixed with Flemming. After Flemming fixation the membrane was distinctly colored by methyl green, and somewhat less so by acetocarmine. Many times the stained membrane was almost as dark as the endosomes. A similar affinity for basic dyes was observed in the nuclear membrane of nuclei fixed in Flemming and Champy in permanent slides. Among the simple fixatives, osmium showed a very distinct tendency to cause the membrane to become basophilic, and chromic acid, also, gave the membrane a less marked tendency to be stained with haematoxylin. Baker (1933) suggests that the chromic acid causes an increased affinity of tissue for haematoxylin by a mordanting action. This is even more marked with mitochondria and other cytoplasmic structures. In view of the mordanting effect of chromic acid which has been postulated, the writer is inclined to explain the basophilic affinities of the Flemming-fixed nuclear membrane as being caused by

osmic acid. It seems probable that it depends on the nature of the additive compounds formed by osmium and the substances composing the nuclear membrane, which Baker (1933) believes are formed.

Fixation of the peripheral zone causes very profound changes in its appearances. The strong alcohol-acetic-sublimate fixatives precipitate the peripheral ground-plasm, almost invisible in the living nucleus, in fine particles which gather in irregular, flocculent masses. Only rarely is a reticulum formed. In all cases observed, this tendency was most marked in Gilson-Carnoy and Schaudinn, followed by Zenker, Bouin, Carnoy, and Flemming in approximately that order. These effects, although very obvious and easy to observe, probably involve many diverse chemical and physical reactions of fixative and substances comprising the ground-plasm. Certainly the original colloid is greatly altered even in Flemming, when a coarse precipitate is formed which is almost evenly distributed throughout the peripheral region. To what extent the results observed can be traced back to the effects as seen in the observations of the effects of simple fixatives is difficult to say. Those substances which cause the greatest degree of accumulation of particles in flocculent masses in the fixatives studied must be sought in the components of Gilson-Carnoy, Schaudinn, and Zenker, and should be absent in Flemming. No such substance can be found, for there is no single substance present in the first three and absent in the last fixatives. It seems probable that mercuric chloride, present in the vigorous fixatives, does not bring about the formation of the flocculent masses, since when used alone it leaves the peripheral ground-plasm in a comparatively homogeneous condition. Similarly alcohol, which is found in the first two fixatives, did not cause such a flocculent mass of substance to be formed when used alone. Acetic acid, however, when used in higher concentrations, did bring about the appearance in question, and may be the most important factor in causing it in Schaudinn and Gilson-Carnoy. Probably in Zenker fixation, the acetic effect is aided, at least, if not replaced, by the effect of the potassium bichromate, which in several attempts also caused a vigorous precipitation, which tended to be accompanied by the arrangement of the particles in flocculent masses. The action of the acetic may be hindered in Carnoy by the greater proportion of alcohol and by the absence of water. The Flemming effect may be caused by the low percentage of acetic, while in Bouin it is possible that the formaldehyde hinders the acetic effect. Until further work of a more detailed nature can be undertaken, the complete explanation of the effects of the various compound fixatives cannot be satisfactorily explained, and the above must be classed as suggestion rather than explanation.

Lying imbedded in the precipitated peripheral ground-plasm are the

peripheral spherules and granules. The spherules are dissolved, partially or entirely, by most of the fixatives, or are imperfectly preserved. Gilson-Carnoy and Schaudinn appeared to cause the spherules to dissolve, or, perhaps, completely obscured the remnants of the spherules in the mass of ground-plasm. Carnoy caused a partial solution of the spherules. Flemming fixation was accompanied by a loss in refractivity of the spherules, and in the unstained nucleus the spherules were almost obscured. Even Flemming fixation failed to preserve the spherules entirely. In Bouin fixation the spherules disappeared very rapidly, so that in less than a quarter of a minute after the fixative had begun to act the spherules were completely dissolved. With Zenker the spherules also dissolved, but in a very deliberate fashion. When the fixative first came in contact with the nucleus the spherules became less refractive. Then, after from five to eight minutes a diminution in the diameter of the spherules became noticeable. In about ten minutes the spherules were almost completely dissolved and shortly thereafter they were no longer visible. Attempts to understand the action of the compound fixatives with the use of the observations made on the action of the simple fixatives has not been entirely satisfactory in the case of the peripheral spherules. Mercuric chloride, osmium tetroxide, and chromic acid cause the solution of the spherules, as do the high grades of acetic acid. The other fixatives either preserve them imperfectly or leave them in a condition in which they may be partially destroyed by the technical procedures followed during the preparations of permanent mounts. Formalin alone causes a lifelike preservation of the peripheral spherules. In view of the above, it is easy to explain why the various compound fixatives dissolve, or fail to fix, the spherules, for they all contain at least one substance inimical to the spherules. The real difficulty comes in explaining the action of Flemming (Fig. 39), which, although it contains chromic acid and osmium tetroxide, does not dissolve the spherules. This seems to indicate that the spherules are perhaps precipitated by low grades of acetic in an insoluble form, an idea which the study of the action of acetic alone does not entirely controvert. If this is the case, however, it is not clear why the same effect does not occur in Zenker fixation. At present, no explanation can be offered for these results.

Observations on the peripheral granules can be made very briefly. With the compound fixatives, generally speaking, the granules become more refractive during fixation. This is, in the case of the alcohol-acetic-sublimate fixatives, at least partially obscured by the heavy precipitate of the ground-plasm. Careful study, however, almost invariably shows the granules to be lying interspersed throughout the ground-plasm. In all cases the granules are lightly stained by methyl green and acetocarmine.

The fact that the granules are visible, although with difficulty, in the living nucleus, and that the same granules have been observed to become more numerous and refractive during fixation while the peripheral spherules disappear indicates very clearly the fact that there are two definitely different types of inclusions in the peripheral region of the nucleus, confirming the observations of Janicki (1909) and Kudo (1926). This point has been the source of some difficulty in the past (see p. 32). Janicki contended that there were two types of inclusions, one of which dissolved during dehydration. Kudo likewise considered that there were two types of inclusions, one of which dissolved during fixation. Basophilic ones, invisible during life, were believed to appear during fixation to give the peripheral region its characteristic stippled appearance in permanent mounts. Elmassian (1909), Mercier (1910), and Morris (1936) appear to have noticed only one kind of peripheral inclusion, believing that the spherules and the granules were identical. Sassuchin (1936) mentions two types of inclusions distinguishable on the basis of staining reaction, probably referring to the peripheral spherules and granules. Since it has been shown in the present study that (1) the peripheral granules and spherules are both visible in life in favorable nuclei, (2) the peripheral spherules are resistant to staining when present in fixed nuclei, (3) the peripheral granules are smaller than the spherules, more irregular in shape, basophilic, and distributed differently in living and fixed nuclei, and (4) the peripheral spherules have been observed during fixation in the process of dissolving in nuclei in which the granules were clearly visible, it seems that the thesis of Kudo can be confirmed completely. It may be appended that the spherules may dissolve, at least partially, during the preparation of the permanent slides, but that this appears to occur in water and not in alcohol, as suspected by Janicki.

The endosomes, invisible in life or visible as indefinitely outlined shapes, become much more refractive during fixation with all compound fixatives. They are invariably precipitated almost instantaneously and undergo no further visible changes. The amount of shrinkage could be determined in but one nucleus in which the endosomes were distinctly visible in the living state, and therefore was not typical. This nucleus, fixed in Zenker, showed very little shrinkage, and the endosomes underwent no measurable shrinkage. The endosomes, homogeneous and hyaline after all fixatives when comparatively small, were frequently divided into two types of substances in the nuclei in which large endosomes prevailed. In this case the cortical portion of the endosomes was more dense and refractive, and the inner portion more dispersed and less refractive. The inner substance was sometimes collected in vacuoles in

the largest endosomes. The endosomes were invariably basophilic, staining rather deeply with methyl green and acetocarmine. In nuclei fixed with Zenker the cortical and inner types of substances were particularly well shown, and the denser substance appeared to be more basophilic, even in the temporary slides stained with acetocarmine. Endosomes fixed in the elongate strand condition showed no inner structure. Since there were so few visible differences in endosomes fixed by the various simple fixatives it is not possible to speculate on the part the simple fixatives play in bringing about the precipitation of the endosomal substances.

Because of the increased opacity of the peripheral material resulting from precipitation of the ground-plasm, the central region could be studied in detail in but a few favorable cases. The central ground-plasm appeared to be precipitated as a homogeneous region by Gilson-Carnoy and Zenker. Carnoy-fixed nuclei had a finely granular, amorphous, or indistinctly reticular central region. Schaudinn contracts the central region into a dense mass of rather coarse particles in which a hyaline body may occasionally be seen. Flemming, like Schaudinn, induced a considerable amount of shrinkage of the central region, which although less marked than in Schaudinn, separated the two ground-plasms at the margin of the peripheral region. The central ground-plasm precipitate was rather coarse. When fixed in the late kinetophase or early interphase condition the Flemming-fixed nuclei frequently show well-preserved hyaline bodies at one pole of the central ground-plasm (cf. Figs. 38 and 39). The hyaline body, invisible in life, is homogeneous and refractive in the fixed nuclei, with smooth outlines. It was always found at one pole of the ground-plasm, and with some practice could be distinguished from the endosomes without staining with methyl green or acetocarmine. In stained nuclei it was invariably more slightly basophilic than the endosomes. The ground-plasm was always acidophilic, although during early interphase, when the ground-plasm lay near one pole of the nucleus with the hyaline body attached to it, small granules could be seen which appeared to be slightly basophilic. The centriole could be seen so rarely in nuclei immediately after fixation that no systematic observations were attempted for it. As with the endosomes the central region presents so few differences in appearance which are characteristic in the studies with simple fixatives, it is impossible to speculate concerning the part the various simple fixatives play in bringing about the action of the compound fixatives. The hyaline body, however, can be analyzed to a certain extent. It is fixed by osmium, and is visible after fixation with alcohol and mercuric chloride. In the case of alcohol and mercuric chloride, the precipitated hyaline body appears to be readily soluble in

water, for it disappears during the preparation of permanent slides. In the compound fixatives, it was seen only in nuclei fixed with Schaudinn and Flemming. With Schaudinn fixation, it is not insoluble, and so disappears during the after-treatments leading to the preparation of permanent slides. In Flemming-fixed nuclei, however, it is present in permanent mounts. This parallels closely the observations made with simple fixatives. It would appear that acetic acid is distinctly inimical to the precipitation of the hyaline body in larger quantities, for in Gilson-Carnoy, containing the alcohol and sublimate necessary to precipitate it in a soluble form it was never seen. It was never found in Zenker-fixed nuclei, which also suggests that it may possibly be destroyed by potassium dichromate. Not enough work was done with fixation resulting from potassium bichromate alone to make a comparison on this point, although a hyaline body was never seen in dichromate-fixed nuclei. It may be said that of the various simple fixatives tried, osmium is the only one to precipitate the hyaline body in an insoluble form, and that compound fixatives which contain osmium are most likely to preserve it in a recognizable form. In several nuclei fixed with Champy the hyaline body was present, which serves to strengthen this supposition.

XII. NATURE OF THE ELEMENTS OF THE TROPHIC NUCLEUS

IT APPEARS from the preceding descriptions that there are a number of distinctive elements occurring in the nucleus of *E. blattae*, visible in living or in fixed amoebae. In order to summarize the results of the observations made on the various types of material, the following conclusions and inferences may be given.

Nuclear membrane.—The nuclear membrane is a homogeneous structure in both living and fixed conditions. In rare cases after Gilson-Carnoy fixation it may appear superficially to be bilamellar in structure, but this condition is encountered so rarely that it may be assumed with safety that it is abnormal, even though it is unaccompanied by definite morphological evidences of degeneration. It is not probable that it is entirely an effect of fixation. The fact that Schubotz (1905) noticed in an unpreserved, dead organism a double nuclear membrane appears to indicate that the bilamellar condition may exist without fixation, and the rarity of its occurrence even in material fixed in Gilson-Carnoy suggests that it was not the fault of the fixative entirely. A stratified appearance may be observed in almost all nuclei either in life or in fixed condition. This appears to be due entirely to the diffraction of light, and is not considered to be an indication of structural lamination. In no case has a

delicate membrane between the cytoplasm and the nucleus, such as that described by Bütschli (1878), been observed. No suggestions concerning the structural basis of Bütschli's description can be made at this time. In many amoebae the nuclear membrane is drawn out into a beak-like projection, as observed by all previous investigators. This appears during the late division stages as a remnant of the inter-nuclear strand, and gradually disappears after division, as stated by Janicki (1909). Janicki's statement that the nuclear membrane is thinner at the locus of the extension appears to be true during the time it is prominent, but as it becomes smaller it forms a small rounded prominence on the membrane in which the wall of the nucleus is thickened. Concerning the striations of the nuclear membrane, described by Sassuchin (1930), who interpreted the striations as canals leading through the nuclear membrane, little can be said at the present. At no time have such striations been seen in bright or in dark field. Fixed nuclei, also, are without such striations, insofar as could be determined with the optical equipment used. In a few nuclei stained with safranin, striations were produced by the arrested diffusion of the stain through the membrane, but these do not appear to be analogous to those described by Sassuchin. In its reactions to stains the nuclear membrane is almost invariably acidophilic, although it acquires a slight affinity for basic dyes after treatment with osmic fixatives. In the latter case this is most marked with haematoxylin fixation, but may also be noticed after staining with methyl green, acetocarmine, and to a slighter degree, with safranin. The basophilic properties are apparently the direct result of the formation of additive compounds with the osmium, or possibly with chromic acid occurring in the fixative and the substance of the membrane. The fact that all osmium fixatives used gave this effect strengthens the view that it is osmium which forms the additive compounds.

The nuclear membrane yields typical protein reactions to the various treatments to which it was subjected. It was precipitated in an insoluble condition by all of the simple fixatives tried, except, possibly, with dioxane, which appears to have caused a partial dissolution of the membrane. It was slightly soluble after fixation in dilute acetic acid, in which case heat apparently aided in the precipitation, since hot acetic gave a more complete precipitation than cold acetic among the lower grades. In its general reactions it closely resembled the complex cytoplasmic proteins. That it is unlike any of these appears certain, although the difference may conceivably be a physical rather than a chemical one. It is interesting to note that the membrane never lost its characteristic hyaline appearance even in the most vigorous fixatives. This suggests that the membrane is not a pure protein, or that it may possibly be a gelled

colloid of very dense structure. With the present knowledge of the physical condition of the precipitated proteins and protein compounds, no more definite conclusions can be drawn.

Peripheral ground-plasm.—In the living state the peripheral ground-plasm is hyaline and clear, containing numerous peripheral spherules and peripheral granules. Bütschli described it as a finely granular-reticular region. His work was done with living nuclei only. The reticular appearance which he mentioned was not demonstrable in the living nucleus. The reticular appearance frequently seen in fixed preparations is thought to be the result of fixation shrinkage, and a general relationship of the size of the interstices of the reticulum and the amount of shrinkage of the ground-plasm appears to support this view. Baker (1933) also supports the view that the reticular appearance of the karyolymph in metazoan cells is caused by fixation. He says (p. 15), "His [Tellyesniczky] reasons for disbelieving in the meshwork in the nucleus were (1) that it is invisible during life; (2) it does not appear after fixation with those fixatives that are not protein precipitants; (3) that it does appear after fixation with protein precipitants; and (4) that in appearance it resembles a protein coagulum. . . . There seems little doubt, however, that the ground substance of the nucleus is structureless, and that the network or spongework is an artifact produced by fixation." Sharp (1934) appears to agree with Baker on this point. After most fixatives the peripheral ground-plasm is acidophilic, but after Zenker fixation, there is a much heightened affinity of the ground-plasm for the basic dyes. This is noted also, although to a somewhat slighter degree, after fixation in Flemming and Champy. This may possibly be traced to the chrome compounds which may have a mordanting effect, especially for haematoxylin.

It may be mentioned here that in no case do the peripheral and central ground-plasms appear to be continuous. They may be in contact for a lesser or greater portion of their margins, but invariably present some visible signs of differentiation. This may appear as a physical difference in the precipitated particles with respect to size, as in Gilson-Carnoy fixation, in which the central ground-plasm is composed of very fine particles and the peripheral ground-plasm of coarser ones. It may appear as a difference in stainability, as in Carnoy, when the peripheral net is acidophilic, and the central net somewhat basophilic, or in the size of the meshes of the nets, as is the case in Flemming, Carnoy, Schaudinn, etc. All of these point to the conclusion that the peripheral and central ground-plasms are to be considered as distinct substances, differing in physical, if not in chemical, nature, and in no case are to be considered as continuous, as suggested by Elmassian (1909).

The peripheral ground-plasm appears to be of protein nature, since it

is precipitated with protein precipitants, and shows other typical protein reactions. Its failure to react with basic dyes suggests that it is not combined with nucleic acid in the form of a nuclein or a nucleoprotein. This interpretation is further suggested by the fact that the peripheral ground-plasm is very rapidly dissolved with pepsin-hydrochloric acid. At present it appears impossible to come to any conclusions concerning its chemical nature, but it is inferred that the ground-plasm is a rather complex mixture of substances, with the basic material being a protein which is normally in the gel phase during interphase, and appears to shift to the sol phase during kinetophase. This protein material appears to be similar to, but not identical with, the substances of like nature occurring in the cytoplasm, for there is a consistent visible differentiation between these two protein substances.

Peripheral spherules.—In the living nucleus the peripheral ground-plasm contains large numbers of highly refractive spherules. These are clumped at one end of the nucleus during the early interphase where they frequently remain long after the other nuclear elements have regained their original concentric organization. During fixation the spherules react in extremely diverse ways. With some, Bouin's, for example, the spherules rapidly dissolve, with others, such as Zenker, they dissolve very slowly, and in a few cases they are not changed in appearance, as in formaldehyde. They are quite resistant to staining with all stains. In safranin preparations, following a Flemming fixation, the remains of the spherules are colored a very light pink shade. This is the only known case where they react positively with a basic dye. Occasionally they are lightly stained with acid dyes. It is not impossible that these observed staining reactions are actually accumulations of stain at the surface of the spherules. The spherules have been the source of much difficulty in the past. Schubotz (1905), who was the first to describe them, was uncertain as to whether or not they were visible in the fixed nucleus. Elmassian (1909) believed that they were identical with the granules he observed in the fixed nucleus. Janicki (1909) stated that they were soluble in alcohols and appeared only in preparations which had undergone rapid dehydration and then were present in a partially dissolved condition. Mercier (1910) described them from the living nucleus but did not definitely link them with the granules seen in fixed nuclei. Kudo (1926) stated that they dissolve in the process of fixation and are replaced by chromatin granules in the fixed nucleus. Morris (1936) stated that the refractive spherules in the living nucleus were identical with the chromatic ones of the fixed and stained nuclei.

On the basis of the observations made on the fixation of nuclei, it seems evident that the peripheral spherules are soluble in some fixatives,

as stated by Kudo, but not in all of them. In those fixatives in which they are not dissolved, some preserve them well, and others do so but poorly. The spherules do not appear to be soluble in alcohol, as suggested by Janicki, but are actually preserved, although not perfectly, by absolute alcohol. It appears that during the dehydration process the spherules, if partially destroyed, are dissolved by the water rather than by the alcohol. It can be stated without reservation that the spherules and granules are not identical. In favorable cases both are visible in the living nucleus, and in a few cases, both are visible in fixed nuclei. When they are both present in the fixed nucleus, the spherules are resistant to staining, while the granules are basophilic. It seems evident that the conclusions reached by Kudo and Janicki were essentially correct.

In their reactions to most of the techniques tried, the peripheral spherules gave results which were rather dubious. They were coagulated by heat, it appears, since in some cases the fixation of the spherules was improved by heating the fixative. They were also partially coagulated by alcohol. Although seemingly partially soluble in glacial acetic acid, there was no evidence to show that they were soluble in dilute acetic acid, and appeared to be insoluble in alcohol following treatment with dilute acetic acid. They were insoluble in picric acid and soluble in chromic acid. Mercuric chloride left them in a rather insoluble condition in saturated solution, insofar as water and alcohol are concerned, but the spherules dissolved in the saturated sublimate solution if left in it long. These reactions do not fit perfectly with any type of chemical substance with which the writer is familiar. In general, however, they appear to show some relationship to the phospho-globulins, but until more information is available they cannot be placed in this group. It is interesting to observe that if they should belong to some chemical group similar to the phospho-globulins they might serve as a source of paranucleic acid and albumins, and thus supply material for the rest of the nuclear elements. Janicki (1909) has suggested, without offering any significant evidence for its support, that they represent a reserve food supply for the nucleus. If the spherules are acting as a source of phosphorous-containing compounds to be utilized in the production of nucleic acid this would support his ideas. It is interesting in this respect, to observe that during the early precystic development, when the spherules are disappearing (see p. 114), there is a distinct increase in the amount of nucleic acid present in the nucleus, as measured by the Feulgen nucleal reaction. There is no evidence, however, that the appearance and disappearance of the Feulgen-positive substances in the central region of the nucleus during division in the trophic stage is aided in any way by the refractive spherules.

Peripheral granules.—Although never prominent, the peripheral granules may be observed in favorable living nuclei. They are usually obscured partially or wholly by the peripheral spherules. In fixed nuclei the granules are basophilic, but remain uncolored by the Feulgen reaction. Schubotz (1905) reported that they were colored orange in Flemming triple preparations, but in such preparations made for this study they were colored a light red, indicating basophilic properties.

Up to the present time there is little evidence to indicate the function of the peripheral granules in nuclear activities. Janicki (1909) suggested that the peripheral granules served as a supply for the endosomes and that the endosomes grew by the addition of basophilic substances from the granules to their surface. The present study has failed to gain any evidence either for or against this interpretation of the granules' function.

The peripheral granules are precipitated by nearly all reagents used. They were poorly preserved by low grades of alcohols and acetic acid, and osmic tetroxide fixation leaves them in a somewhat indeterminate state. They are digested in pepsin-hydrochloric acid and are soluble in 10 per cent solutions of sodium and potassium chloride. They are basophilic, but less so than the endosomal material. In their general reactions they appear to be closely allied to the nucleins. The uncertain knowledge of nucleins, due to the great variation in their chemical make-up, makes it impossible to go beyond this point in their analysis at this time.

Endosomes.—In the living nucleus the endosomes cannot be studied, as they are visible as indefinite shadows, or wholly invisible. Fixed nuclei are characterized by more prominent endosomes. As they are basophilic they show up well in permanent preparations. In haematoxylin or safranin they stain very dark, and after strong alcoholic fixatives, sublimate fixatives, or Zenker, the inner structure occasionally becomes apparent. The larger endosomes present a typical appearance. There is a dark-staining region in the cortical zone, which represents a shell of basophilic material. This contains a more lightly-staining substance which may fill the whole interior of the endosome, or be lodged in vacuoles. In many cases a central basophilic granule lying at the very center of the endosome can be observed. Janicki (1909) observed that large endosomes are frequently built as vacuolated structures, and Mercier (1910) and Kudo (1926) observed similar structure in some endosomes. Morris (1936) pointed out that there was usually a basophilic cortex and a more lightly-staining interior. The central granule of basophilic material does not appear to have been mentioned, and its significance is not understood. The dual nature of the endosomal material seems quite evident, although all of its implications are not yet understood. The lightly-staining material may be formed from the basophilic portion, which may

account for the growth of the endosomes during the late interphase period, but the evidence for this point is very slight. There is no reason to believe with Janicki (1909) that the endosomal growth during interphase occurs as a result of the endosome taking up substances from the central region, since in reaction to fixatives and stains the substances are distinctly different. Although intensely basophilic, the endosomes do not react with the Feulgen reagents at any time during interphase or kinetophase.

The endosomal variation in shape, size, and number is very striking. Shape varies from elongate to the predominantly spherical types, with various intermediate shapes. The number of endosomes varies from about five or six to twenty-four or more. Morris (1936) points out that the endosomal number approximates the chromosomal number. The wide range of variation and the fact that there is some indication of a reduction of endosome number during the late interphase period suggests that this similarity between average number of endosomes and approximate number of chromosomes is coincidental rather than indicative of a true correlation.

The cortical, basophilic, portion of the endosomes is well preserved by mercuric chloride, chromic acid, and 10 per cent acetic acid. It is less well preserved by alcohol, osmium, and formaldehyde, and appears to be irregular and doubtfully preserved in glacial acetic acid. The substance is rather resistant to digestion when put in pepsin-hydrochloric acid solution, but is broken down after some time. It is soluble in the 10 per cent solutions of sodium and potassium chlorides. These reactions suggest that we have to do with a nucleoprotein. The poor preservation of the endosomes in stronger acetic acid solutions is believed to be primarily the result of the action of the acetic on the inner, more lightly-staining material. The basophilic part of the endosomes appears to agree in all particulars with the expected reactions of nucleoproteins if this view is maintained.

The inner endosomal material is less easily determined. It is preserved very well in picric acid, chromic acid, and mercuric chloride, and less well by alcohol, glacial acetic acid, osmium, and formaldehyde. It is preserved in dilute acetic acids, however. It seems to be dissolved by 10 per cent solutions of sodium and potassium chlorides, and is rapidly attacked by pepsin-hydrochloric acid digestion. It seems possible that it may represent a product of the disintegration of the basophilic outer portion of the endosomes, which might suggest that it is a nuclein of some kind. However, the acetic acid reaction argues against this interpretation. Mann (1902) showed that nuclein is precipitated by acetic acid, which will not account for the poor preservation of this material by glacial

acetic acid. The reaction of protalbumose is more nearly identical with the observed reactions. Protalbumose is precipitated by alcohol, but in a soluble form, according to Mann, accounting for the comparatively poor fixation with alcohol. Osmium and formaldehyde might be expected to fix protalbumose poorly according to Mann's table. The fact that protalbumose is soluble in high grades of acetic, but insoluble after treatment with the low grades also suggests that this may be the material found in the center of the endosomes. Like many other types of substances, protalbumoses are well preserved by chromic acid and sublimate. Artificial digestion experiments support this view. On the basis of the present information, the writer is inclined to the opinion that the inner endosomal substance is composed of protalbumose, or some closely allied substance, although much more work is necessary before any definite conclusion is possible.

Central ground-plasm.—The ground-plasm of the central region is transparent and structureless in living nuclei. Schubotz (1905) believed that he could see a fine reticulum in the central region of living nuclei, but in the material studied for this investigation, this was not observed. The central region, like the peripheral region, is essentially acidophilic in its reactions to stains, but in other respects it is distinctly different. It appears that the acidophilic reaction of the central ground-plasm reaches its height in the middle of the interphase period, and the substance becomes more basophilic as division approaches, although it never becomes intensely so.

Many amoebae do not react positively to the Feulgen test for nucleic acids. *E. blattae* has been thought to belong to this group. Morris (1936, p. 230) says, "Although tried in many variations the Feulgen reaction gave uniformly negative results, although ciliates on the same slides stained well." The results of this study do not confirm his results, except during the interphase of the trophic stage. Sassuchin (1936) also reports that the nucleus of *E. blattae* does not react with the Feulgen reagents, but suggests that at other parts of its life cycle, it may show a positive reaction. As Sassuchin suggested, the nucleus does not remain negative to the Feulgen reaction throughout the life cycle. Although invariably negative to the Feulgen reaction during the interphase, the central region gradually becomes somewhat diffusely positive during the early division stages, and the chromosomes which are formed from the substance of the central ground-plasm are distinctly positive. During the reorganization of the nucleus after division, the chromosomes break down, and the formation of a new central region from the chromosomal substance is accompanied by the gradual disappearance of the positive reaction with the Feulgen reagents. While the Feulgen reaction is quite

possibly not specific for nucleic acids, it is the best test which we have at present for them. The fact that after fixation with alcohol the nucleic acids are precipitated, but in a soluble condition, and that nuclei fixed in alcohol showed chromosomes which were poorly preserved and failed to react well with Feulgen reagents, although the techniques used were the same as those which gave good reactions during similar division stages after fixation in compound fixatives, supports the idea that the reacting substance was nucleic acid. The results of fixation of the chromosomes and the central region during stages just prior to or just after division in various simple fixatives shows that the Feulgen-positive substances and the chromosomes reacted as one would expect nucleic acid to react according to the table shown by Mann (1902) and the similar work of Fischer (1899). In view of these facts it is believed that the Feulgen-positive material in *E. blattac* is a thymo-nucleic acid, and that the nucleic acid content of the central region fluctuates during the division cycle, reaching a peak during the anaphase-like stage while the chromosomes are clumped at the poles of the dividing nucleus, and disappearing during the reconstruction of the nucleus. It appears probable that the disappearance of the nucleic acids involves a combination of the nucleic acid into a nuclein or nucleoprotein. During the interphase the substance composing the central ground-plasm appears to react, in general, similarly to nucleoproteins. It shows considerable resistance to digestion with pepsin-hydrochloric acid, and in other respects appears to show some similarities to nucleoproteins. Although it usually shows affinities for acid dyes, it seems probable that the central ground-plasm during the interphase stage is composed of a nucleoprotein, different, certainly from the one noticed in the endosomal cortex. It is most probable that the nucleoproteins of the central ground-plasm are associated with various other compounds, of which the majority appear to be of a protein nature.

Imbedded in the central ground-plasm are delicate granules in some nuclei. They are quite invisible in the living nucleus, but the regularity with which they appear in fixed nuclei in which diverse techniques have been used suggest that they are not artifacts. Schubotz (1905) observed such granules, and from artificial digestion experiments came to the conclusion that they were the only chromatin in the nucleus. This does not agree with the results of the present study with respect to artificial digestion experiments (see p. 32), and it is possible that Schubotz used different concentrations of hydrochloric acid or pepsin. Janicki (1909) also described chromatic central granules. Kudo (1926) states that the central region usually contained no chromatin granules except during early division stages, when he believed that the chromatin granules may migrate into the central region. In the material studied it seems probable that, as Kudo observed, the chromatin granules occur in the central

region only in stages approaching division and that the central region granules are peculiar to early kinetophase nuclei. No evidence in favor of the migration of peripheral granules into the central region could be found, however, and it seems probable that the granules are associated with the chromosomes, for when they first appear the chromosomes assume a beaded appearance. These granules are somewhat basophilic, but not distinctly so, and are not specifically positive to the Feulgen test. They appear to be equivalent to the granules described on the chromosomes of *E. disparata* by Kirby (1927). It is possible that these granules represent the collected nucleoprotein of the central region, but although early observations point to this inference, not enough work has been completed on nuclei in this stage to make a definite conclusion possible.

Hyaline body.—After Flemming or Champy fixation a hyaline structure may be found in the central region. It was found in fixed nuclei only, but Sassuchin (1930) reports having seen a body in the central region of living nuclei which he believes is identical with the "karyosome" of Janicki (1909). Similarly Bütschli (1878) reported that there was a dark body visible in the central region of some nuclei. Mercier (1910) and Kudo (1926) did not observe this structure in living or fixed nuclei, but Janicki (1909) gives a good description, although his figures are not very clear. According to Janicki it is homogeneous and lies at one pole of the central region. It is almost constant in position. It initiates karyokinesis by its division and the formation of a spindle from its substance. Although his description of its position and general appearance seems to fit the hyaline body, no relationship to division as reported by Janicki has been observed. He termed the structure a karyosome, but it seems that since it does not appear to be homologous structurally or functionally to the structure ordinarily called a karyosome in other amoebae, another term should be used.

In nuclei stained with safranin the hyaline body is most prominent. It is much more difficult to distinguish in haematoxylin preparations. It is less basophilic than the endosomes and its constant position at one pole of the central zone is so characteristic that it can usually be recognized easily. It is most evident during the late kinetophase and early interphase period, when it lies at the pole of the dedifferentiating chromosomes as a distinctly basophilic body. During later interphase development it is no longer basophilic, and finally disappears altogether. No spindle has been observed in the dividing nuclei, and a division of the hyaline body has never been witnessed. It first appears when the chromosomes are clumped at the poles of the nucleus, and at the present time its mode of formation is wholly unknown. It appears to be associated with the reorganization of the nucleus after division.

Concerning its reactions with simple fixatives, it is interesting to note

that it may possibly consist of a material closely allied to a protamine. Protamine is precipitated in an insoluble form by chromic acid and mercuric chloride, according to Mann (1902). It is not precipitated by acetic acid nor formaldehyde. It is precipitated in a difficultly soluble form by alcohol. The hyaline body is found after mercuric chloride fixation in prepared mounts. It is observed in cells immediately after fixation in alcohol, but not after washing for long periods of time. It is worthy of note that while protamines are insoluble in absolute alcohol, they are soluble in the lower grades. It was not observed after acetic acid fixation, nor after formaldehyde nor chromic acid. In osmium-fixed material it is incompletely preserved. The absence of the hyaline body in chromic acid material seems to deny that it is a protamine, but it may be pointed out that while it is incompletely preserved by osmium, it is well fixed by the osmic-chromic combination of Flemming's fluid. This may indicate that the chromic acid had some effect on the hyaline body during Flemming fixation.

It is interesting to observe in this connection that the hyaline bodies appear to be very closely associated with the chromosomes during the reconstruction of the central region after division. This is accompanied by a definite shift in the reaction of the substance composing the chromosomes in the staining reactions. Up to the time of the appearance of the hyaline body the chromosomes react positively to the Feulgen test. While it is present this positive reaction is lost, and when it disappears, the central region is composed of Feulgen-negative substance. That protamine has a strong affinity for nucleic acids has been known for a long time, as pointed out by Mann (1902). It is a characteristic substance in the sperm of lower animals, where it appears to be connected with the development of large amounts of nucleic acid found in the mature sperm. It is suggested by the above that the hyaline body may possibly have some similar functional importance in the nucleus of *E. blattae*, if it is indeed composed of a substance resembling protamine. It may be associated with the reduction in nucleic acid content of the chromosomes during their dedifferentiation. This may account for the shift in stainability of a hyaline body, as the nucleic acid from the chromosomes forms a basophilic compound with the substance composing the hyaline body.

XIII. NATURE OF THE CHROMATIN

THE EXACT definition of chromatin is somewhat uncertain at the present time. It has altered a great deal in its significance since the term was first coined to indicate the dark-staining material in the nucleus. It became increasingly apparent as the nuclear activities were more completely understood that the dark- and light-staining materials might be more

closely related functionally than was originally believed. The concept of oxychromatin and basichromatin, developed by Flemming, defined the dark-staining material as basichromatin because it stained with basic dyes, and the light-staining material oxychromatin because it stained with acid dyes. There was an attempt to distinguish between achromatin, the so-called "linin" or nuclear reticulum from the oxychromatin, but this was very indefinite, and, as stated by Sharp (1920, p. 64), "As used by many writers the term oxychromatin includes also the linin, so that in much of the cytological literature linin and oxychromatin are more or less interchangeable terms, while "chromatin" refers to the basichromatin."

Bělař (1926) summed up some of the objections to the older conception of chromatic elements in the nucleus. He found the term "achromatin," as distinguished from oxychromatin, undesirable. He says (p. 243), "Es erscheint daher immer nach vorderhand zweckmässiger, solche 'achromatischer' Strukturen, denen vitale Realität nicht aberkannt werden darf, einfach als Differenzierung der amorphen Kerngrundsubstanz zu bezeichnen." He expresses the opinion that the term chromatin is a functional rather than a chemical one. Thus he says (p. 243), "In denjenigen—noch dazu recht seltenen—Fällen, wo an chromatischen Strukturelementen, deren vitale präexistenz feststeht, eine Unterscheidung von acidophiler und basophiler Substanz möglich erscheint (z. B. die sogenannten Chromomeren und das chromatische Chromosomen 'skelett'), sei jedoch daran erinnert, dass der chromatin begriff letzten Endes ein *morphologischer* ist und dass wir nicht selten aus neutral oder gar acidophil reagierenden Strukturen Chromosomen hervorgehen sehen." Following this line of thought the chromatin cannot be analyzed chemically, since (p. 241), "Eben diese morphogenetische Analyse zwingt uns aber andererseits, in vielen Fällen Strukturen, die sich färberisch entgegengesetzt oder neutral verhalten, ebenfalls als Chromatin zu bezeichnen, sobald nämlich der Nachweis erbracht ist, dass sie genetisch mit den Chromosomen zusammenhängen. Die Ausdrucksweise 'chromatinarmer' und 'chromatinreicher' Kern is somit als Unfug zu bezeichnen." Thus Bělař's concept appears to have diverged far from the classical basichromatin-oxychromatin concept.

Gutherz (1927) differs in his interpretation of chromatin. He says (p. 332), "Im folgenden wird 'Chromatin' ausschliesslich zur Bezeichnung basophiler, d. h. mit basischen Färbstoffen elektiv färbbarer Substanzen innerhalb des Zellkernes bzw. in den ausgebildeten Chromosomen verwendet werden. . . . Der Begriff 'Oxychromatin' scheidet definitionsgemäss für unsere Betrachtung aus." He, thus, disposes of the term oxychromatin by considering only basophilic material as chromatic.

Sharp (1934) refers the question of the nature of the difference

between basic and acidophilic materials in the nucleus to either a physical difference, a chemical difference, or a combination of the two. He says (p. 55), "It has been suggested that if there are two elements, chromatin and linin, they are not so distinct morphologically as the earlier workers supposed, the chromatin existing rather as a thin fluid impregnating the linin substratum. The chromatic lumps are often not sharply set off from the rest of the thread but taper off gradually. In such cases it has been found that purposes of cytological description are well served by the conception of a reticulum composed of a single complex substance which stains variously in different regions and at different stages of the nuclear cycle, according to the size of the strands, their physico-chemical state, and the technical procedure employed. This one substance is loosely spoken of as chromatin, but because of the long application of this term to a supposedly distinct component of the reticulum it is advisable to use Lundegardh's (1910) term *karyotin* for the reticular substance as a whole. Only future research can decide whether karyotin ('chromatin' in the wide sense) is a true chemical compound or a looser combination of two or more constituents, only one of which is 'chromatin' (in the narrow sense)."

In the present study, it would have been possible to term chromatin, following Guthertz's basophilic concept, to apply to the endosomes and peripheral granules only, except for the very light basophilic reaction of the chromosomes, which might be included as chromatin-containing structures. By using Bělař's functional definition, the comparatively non-basophilic material of the central region would be termed chromatin, since it is from this substance that the chromosomes are formed. The broad sense suggested by Sharp would lead to the whole nuclear contents, except, perhaps, the peripheral spherules, to be considered as chromatin. Of course, not all of the points of view expressed about chromatin have been cited, and still other interpretations might be made, based on other uncited and unmentioned opinions. This great confusion makes it quite difficult to use the term chromatin until some future studies and discussions have unified the concept.

The variety of nuclear elements in this organism makes it doubly difficult to reach a definition of chromatin. It was decided that some attempt to study the nucleus with the so-called specific chromatin tests might lead to a possible solution. In this attempt to determine the relation of chromatin and the various nuclear elements the following tests were applied: the Feulgen nucleal reaction, acidified methyl green, 10 per cent NaCl solution, comparison of the action of 10 per cent KCl and CaCl₂ with the NaCl solution, and artificial digestion experiments.

The development of a technique by Feulgen and Rossenbeck (1924)

which could be applied to cytological material for the demonstration of free nucleic acid made it possible to determine the distribution and relationship of nucleic acid to the nuclear elements. It has been pointed out by numerous investigators that the Feulgen nucleal reaction is not to be considered as specific for nucleic acids. There is the substance plasmogen, in the cytoplasm, which gives a positive reaction, unless removed by solution in 95 per cent alcohol, and in addition there are other compounds which may occur in the cytoplasm of various types of cells which will react positively. In spite of all these various objections the Feulgen nucleal reaction is the best that we have at the present time, and the writer is of the opinion that for substances within the nuclear membrane it is most advantageous to consider it specific for nucleic acids until further microchemical tests have made it possible to refine our present concept. For that reason, and with these reservations, the writer has used the terms nucleic acid, and Feulgen-positive material interchangeably. Only substances within the nucleus are considered here, and the positive reaction in the cytoplasm is not referred to. By comparing the intensity of the reaction of the nuclear substances with the Feulgen reagents as indicated by a deeper violet color, it was possible to estimate comparative amounts of nucleic acid present at different periods. The writer wishes to point out that although this is distinctly a qualitative test, it may yield a rough quantitative measure of a substance or group of substances which, for lack of a more accurate term, have been called nucleic acids, not in the strict chemical sense, but in a loose cytological sense. Observations of the effects of the nucleal reaction at various times during the trophic phase of the life cycle have revealed a distinct cyclical variation in the amount of nucleic acid present in the nucleus of *E. blattae*, associated with the division cycle.

During the middle and late interphase periods there is no reaction demonstrable in the nucleus of *E. blattae*. Because of the comparatively short time required for the nucleus to complete its division, this includes well over 95 per cent of all the nuclei seen in the present study. At this time the whole nucleus, following the standardized Feulgen technique cited previously, gives no reaction which is visible with the Feulgen reagents. The nucleus stains with the acid dyes. As the kinetophase approaches, however, the central region of the nucleus begins to show a very light violet reaction. This was so weak that it was not noticed in the first preparations, and it was not until later stages were studied in which a more decided reaction was obtained that the earlier stages were found to be positive. As the kinetophase advances the original diffuse reaction becomes more intense and the anlage of the chromosomes appear. At this time the chromosomal strands do not appear to be positive to a greater

degree than the surrounding material. Granules appear in the central region at this time, and the chromosomal strands, the granules, and the ground-plasm are all positive to approximately the same degree. Following the so-called "dedifferentiated" stage of nuclear division, when a reorganization of the peripheral material is completed, the amount of nucleic acid present as indicated by an increased deepening of the Feulgen reaction increases quite rapidly. As the chromosomes migrate to the poles of the nucleus they enter a massive condition in which the granules cannot be distinguished readily in most nuclei. At this time they are colored most intensely by the reaction. Even at this time they are by no means dark, and, as compared to the macronucleus of the ciliates *Nyctotherus ovalis* and *Balantidium praenucleatum* which occur on the same slides, the coloration would be termed quite light.

Until the chromosomes begin to dedifferentiate the Feulgen reaction continues to be comparatively intense. As soon as the chromosomes begin to fuse together and form the new central region of the daughter nucleus, the amount of nucleic acid present appears to decrease rapidly. At this time the hyaline body, lying very closely pressed against the clumped chromosomes, occasionally shows a very light positive reaction. This, however, is not sufficiently regular to permit considering it a normal part of the division cycle. It does, however, normally present a somewhat basophilic aspect at this time. As the chromosomes alter their shape, and finally form a new central region, the violet color disappears entirely. After the endosomes have attained the sinuous strand stage (Fig. 1), the central region no longer reacts positively to the Feulgen test.

In two nuclei of the thousands observed the endosomal material was colored a very light violet shade by the Feulgen reaction. Both of these nuclei were fixed in Gilson-Carnoy. This appears, from its extreme rarity, to be an atypical condition, for in no other case was a positive reaction with endosomal material observed. It is possible, of course, that there is a minimal reaction so slight that it cannot be seen, but with the present development of the Feulgen technique, the endosomes must be considered as negative to the test.

Taken as a specific test for chromatin, the nucleus appears to be wholly lacking in chromatin at times, and never liberally supplied with it. Of course, as with the endosomes, it is possible that there is a subminimal reaction which is not visible, but until further tests are possible this must remain a somewhat dubious possibility. Whatever chromatin normally occurs in the nucleus appears to be present in the central region in normal nuclei. This material, it may be pointed out, is identical with the material which forms the chromosomes, and more or less closely parallels the interpretation of chromatin advanced by Bělař (1926), in-

sofar as division stages themselves are concerned. The long interphase condition would be a period in which the chromosomal material would not be chromatic, however, if the Feulgen test be considered specific for chromatin.

Although approaching a test for chromatin as defined by Bělař, the Feulgen reaction fails utterly as a chromatin test if any other definition than Bělař's be accepted. It does not react with the peripheral basophilic elements, and thus falls short of demonstrating the supposed chromatic elements if any connection between affinity for basic dyes and chromatin be postulated.

To some extent the Feulgen reaction might be considered as specific for the "chromatin in the strict sense" mentioned by Sharp (1934), and would fail absolutely to demonstrate the "chromatin in the wide sense." It fails to demonstrate the former in the interphase state, however, and thus stands in the same relation to Sharp's "chromatin in the strict sense" as it does to Bělař's chromatin determined by its relation to the chromosomes.

As a result of this, the conclusion must be reached that chromatin is not demonstrated specifically by Feulgen's nucleal reaction. Regardless of its value or lack of value as a specific test for nucleic acids, it is not sufficient in itself to determine the chromatic elements of the nucleus, insofar as *E. blattae* is concerned, unless we are willing to admit that during the interphase condition *E. blattae* is lacking enough chromatin to give a positive reaction.

Methyl green has been known as a specific test for chromatin for a long time. It is used as a temporary stain, applied directly to the living organism in a solution acidified with 1 per cent acetic acid. The 1 per cent solution of the dye, which has been widely recommended was found to be too strong for *E. blattae*, and a 0.25 per cent was found to be much more efficacious. The same technique was used for the study of the effects of methyl green as were used for the observation of the immediate effects of fixation.

As the acidified methyl green struck the organism death occurred instantaneously. It was accompanied by a slight swelling of the nucleus. The membrane was more sharply outlined after fixation, and appeared to be somewhat thinner than in life. It acquired a very light green tint. The peripheral ground-plasm was also tinted a light green color at first, but this gradually changed to a violet color, during the first eleven minutes or so, due to the metachromatic effect of the methyl violet present as an impurity in the stain. The peripheral granules became very slightly more prominent during fixation, and were stained a somewhat darker green than the peripheral ground-plasm. The endosomes became more promi-

ment after fixation and were stained a rather dark green. They were approximately as dark as the most intensely stained peripheral granules (Fig. 35). Sometimes in the larger endosomes the cortical and medullar parts of the endosomes could be identified. The central region was not very clear, but appeared to be stained a very light green, approximately the same as the peripheral ground-plasm and the cytoplasm.

Using methyl green as an indicator of chromatic material the chromatin would appear to be centered in the peripheral granules and the endosomes. This coincides with the basophilic material, and would thus be specific for chromatin as interpreted by Gutherz (1927). In spite of this correlation of the methyl green test and the basophilic material, the writer feels that methyl green cannot be accepted as a specific chromatin test in *E. blattae*. Methyl green, being a basic stain, might be expected to demonstrate the basophilic elements of the nucleus, and can be considered no more specific than is haematoxylin, and possibly less so, since the differentiation between the stained and unstained parts of the amoeba are much less obvious. An obvious difficulty is that if we accept the basophilic definition of chromatin we are led to believe that the central region, which forms the chromosomes, are achromatic in the interphase, and contain very little chromatin during division, a condition which would hardly be anticipated from our concept of the close relationship of chromatin and hereditary units as observed in Metazoa. For that reason the writer feels justified in considering the acidified methyl green and other basic dyes as not demonstrating all of the chromatin in the nucleus and therefore not a specific chromatin test.

Zacharias (1881) found that chromatin was swollen, and finally dissolved by a 10 per cent solution of sodium chloride. This method has been used occasionally as a specific chromatin test. It was applied, using the same method as that employed for the study of the immediate effects of fixation, except that because of the greatly increased density of the organism resulting from the dehydration caused by the high concentration of the salt, it was necessary to compress the animal more than was necessary for the study of fixatives.

Except for a slight darkening of all of the components, the nucleus seemed unchanged when the solution was first added. There was a slight swelling of the whole nucleus, but no apparent focus of increase in size. The most careful attempts failed invariably to reveal a swelling of any single component. A few moments after the solution of NaCl was added there was a slight Brownian movement of the peripheral spherules. This continued to a slight degree throughout the remainder of the experiment. The nuclear contents had contracted away from the membrane slightly within three or four minutes. At this time the central region was probably

at least partially dissolved, for it was very clear, and in some cases there were a few peripheral spherules moving into it. The peripheral ground-plasm also went into solution partially, leaving the peripheral spherules and granules visible. The peripheral granules were the first to go into solution, after which the spherules gradually disappeared. It required from 15 to 20 minutes for the peripheral spherules to disappear entirely. After the spherules had disappeared a number of extremely delicate strands were revealed. These had been obscured by the spherules and remained invisible until the spherules were almost completely gone. They were very thin, just within the limits of visibility. These strands were not dissolved in 30 minutes, and later experiments, not involving constant observation, indicated that they were present even after longer periods.

These strands seem to represent the "achromatin" of the nucleus. The possibility that these strands represent the base on which the reticulum is formed cannot be entirely discounted, but the fact that they cannot be seen in the living nucleus makes it impossible at the present time to determine what their nature is. It is possible that they were formed as a result of the treatment with sodium chloride, and represent artifacts. Their minute dimensions and the fact that they are obscured in the experimental nuclei until the spherules are almost completely dissolved make it uncertain whether they could be seen in the living nucleus if present.

The solubility of the components would suggest that all of the remainder of the material is chromatic in nature if solubility in 10 per cent NaCl be considered a test for chromatin. This interpretation would differ widely from the more specific tests, which isolate one or another structure from the remaining nuclear parts. At the present time the writer is inclined to believe that the method used here cannot be utilized as a specific chromatin test, but might possibly be developed as a test for the "chromatin in a wide sense" as used by Sharp (1934). It seems almost impossible at the present time to speculate on the nature of the strands demonstrated by this technique. One obvious possibility is that they may represent the "linin" of the nucleus. In this case solution with 10 per cent NaCl might be developed in *E. blattae* as a means of separating the "karyotin" from the "linin."

Organisms were also exposed to solutions of 10 per cent KCl and CaCl₂ using the same methods. With the potassium solution the results were identical with those obtained with solutions of NaCl (Figs. 40, 41). The same structures were dissolved and fine strands were left in the nucleus at the end of the experiment. The potassium chloride appeared to react a little more rapidly than the sodium chloride, the whole process being completed in about 10 minutes.

Calcium chloride was not at all similar in its effects. As the solution struck the organism the nucleus underwent a great deal of shrinkage, accompanied by a great wrinkling of the membrane and indentation of the nucleus. The nuclear membrane disappeared rather rapidly, and the various components became less refractive. The peripheral spherules became quite indistinct. The central region disappeared when the indentation of the nucleus occurred, appearing to be crowded out of the picture. Addition of water caused the nucleus to swell, but when an indentation had formed, as was usually the case, it remained present. The peripheral spherules were found to be somewhat indistinct and were apparently partially fused. Prolonged action of the original calcium chloride solution did not bring about their solution nor otherwise alter their appearance.

It was concluded from this that the potassium chloride solution was as good for dissolving material from the nucleus as the sodium chloride in solutions of the same percentage. Whether either of them can be considered as specific tests for chromatin in *E. blattae* is extremely dubious, and is contraindicated by most of the modern conceptions of chromatin. On one point it seemed that results of some importance were obtained. In spite of the fact that the peripheral ground-plasm reacts to stains and fixatives in much the same way as the cytoplasm does, and is apparently basically a protein colloid, possibly containing other compounds in it, it reacts very differently than the cytoplasm to the sodium and potassium chloride solutions. This seems to indicate rather forcibly that the cytoplasmic and nuclear proteins are not identical in either physical or chemical states, or both. It was also concluded that calcium chloride could not be used as a nuclear solvent, and did not act in any way that was comparable to the action of sodium and potassium chlorides.

Resistance to digestion with pepsin-hydrochloric acid solutions has been used for many years as a test for chromatin. Schubotz (1905) is the only previous investigator to report the action of this solution on the nucleus of *E. blattae*. His results indicated that the chromatin occurring in the nucleus was restricted to small granules which gathered in the center of the nucleus. The results of the digestion carried on in this study differ somewhat from those reported by Schubotz. A 2 per cent solution of pepsin in 0.5 per cent hydrochloric acid was used at 37° C. By using a warm stage it was possible to keep the experimental amoebae under observation continually.

As the pepsin-hydrochloric acid came in contact with the organism, death was instantaneous. The cytoplasm became much darker and the nucleus was swollen. The nuclear membrane almost disappeared. The peripheral spherules were very rapidly dissolved, as were the peripheral

granules. The peripheral ground-plasm disappeared in from 15 to 20 minutes, leaving only the endosomes and the central zone in the nucleus.

The endosomes became more prominent as the solution first came in contact with the organism. They were more refractive and homogeneous at first. Shortly after this first effect was consummated the endosomes began to alter in appearance. In the center of the endosomes a small refractive granule appeared, while a less refractive area surrounded it. The cortex of the endosomes, like the granule in the center, remained highly refractive. This endosomal structure, coinciding with the structure observed in fixed nuclei, seemed to indicate the early solution of the more lightly-staining inner substance of the endosomes, and a greater resistance of the basophilic outer material. After the inner structure became apparent the endosomes changed but little in the next hour. About an hour and a half after the pepsin-hydrochloric acid was first applied the endosomes showed signs of further dissolution. A distinct increase in the size of the vacuoles was noticed and there was a loss in refractivity of the whole structure. Partial fusion of the endosomes was a further indication that they were beginning to go into solution. By about three hours after the application of the pepsin-hydrochloric acid solution the endosomes were dissolved.

The central region was the most resistant to the digestion experiments. No alteration in the appearance of the central region had occurred in two hours except that the material composing it was precipitated and much darker than in the living nucleus. This change had occurred when the solution first struck the organism, and seemed to be an effect of the acid rather than of the pepsin. After three hours slight indications of disintegration had appeared, but these were very limited in extent.

Accepting the resistance to digestion as an indication of chromatic nature, the central region is the most chromatic region of the nucleus. The cortex and the dark-staining central granule of the endosomes represent the remainder of the chromatin. The endosomes, since they dissolve more rapidly than the central region, seem to be less chromatic than the central region. One of the most interesting points was the rapid solution of the basophilic peripheral granules. This may have been due to their small size as compared to the endosomes, or may possibly indicate a distinct chemical difference in the composition of the granules and endosomes. The writer is inclined to favor the latter interpretation, since the granule lying in the center of the endosomes was of about the same size as the peripheral granules, and persisted for several hours.

It is evident from this that the pepsin-hydrochloric acid digestion does not follow any staining reaction in its effect on the nucleus of *E. blattae*. It indicates a chromatic nature for the basophilic cortex of the

endosomes and a non-chromatic nature for the basophilic peripheral granules. It indicates the chromatic nature of the central region and the non-chromatic nature of the peripheral ground-plasm, both of which are more or less acidophilic in their staining reactions. In this respect it fails to follow precisely the definition of chromatin as presented by any of the previous writers with which the author is acquainted, and as such might be considered as non-specific for chromatin in the accepted sense of the term, whatever that may be.

For aid in comparison of the effects of the various tests that were attempted, a summary of the results may be gathered as follows:

Nuclear membrane.—Invariably negative to all tests, except that it stains with basic dyes after osmium fixation.

Peripheral ground-plasm.—Negative to Feulgen; usually unstained by basic dyes; negative to methyl green; dissolved by 10 per cent sodium and potassium chloride, but revealing a number of delicate strands of material resistant to the action of the solutions; dissolved by artificial digestion very rapidly. Thus the ground-plasm is achromatic in all tests except those with sodium and potassium chlorides, in which it appears to be composed of chromatic and achromatic parts, the latter being represented by a number of delicate strands.

Peripheral spherules.—Negative to Feulgen; usually resistant to all stains, except, perhaps, safranin after osmic fixation; dissolve in acidified methyl green; dissolve in 10 per cent sodium and potassium chlorides; dissolve in pepsin-hydrochloric acid. The spherules are therefore negative to all tests except solution in sodium and potassium chloride, in which they give a positive reaction to the chromatin test.

Peripheral granules.—Negative to Feulgen; stained by basic dyes; stained by acidified methyl green; dissolved by 10 per cent sodium and potassium chlorides; dissolved by pepsin-hydrochloric acid. These are inconsistent, being negative to Feulgen and artificial digestion experiment, but positive to the others.

Endosomes, cortical layer, and central granule.—Negative to Feulgen; positive to all other reactions. They are digestible in pepsin-hydrochloric acid, but very slowly so.

Endosomes, inner vacuoles.—Negative to all tests but solubility in 10 per cent sodium and potassium chlorides, in which they apparently dissolve, and to basic dyes, with which they show a slight reaction.

Central ground-plasm.—Negative to Feulgen, except immediately before and immediately after division; usually negative to basic dyes, but always more basophilic than the cytoplasm; unstained by acidified methyl green; soluble in 10 per cent solutions of sodium and potassium chlorides;

resistant to artificial digestion. The alteration in nucleic acid content as measured by the Feulgen reaction makes it periodically positive and negative to that test. Otherwise the central region is uniformly positive for chromatin. The poor stainability of the central ground-plasm to basic dyes as compared to the endosomes is offset to some extent by the fact that it is somewhat more basophilic than the cytoplasm.

The diversity of the results need not be pointed out. As mentioned above, there are difficulties in accepting any of the above as a specific chromatin test, and the choice of any seems to be left up to the discretion of the individual. Since arbitrarily accepting the Feulgen reaction as a specific chromatin test involves the contention that for the greater part of the interphase period the nucleus is composed entirely of achromatic material, or contains subminimal amounts of chromatin, it seems untenable. That nucleic acid is released from nucleoprotein or some similar material at the time of division appears to be quite probable in the case of *E. blattae*. That this same nucleic acid is recombined into more complex nucleoproteins or similar substances is an almost inseparable corollary, to account for the disappearance of the Feulgen-positive material after division. The close relationship of nucleic acid and nucleoprotein when considered from a morphological and functional point of view make it undesirable to differentiate between them, calling one chromatin and the other achromatin. Such functional inconsistencies may be expected from any concept of chromatin as a static chemical compound.

Similarly it seems undesirable to interpret all of the basophilic as chromatin and none of the acidophilic material as chromatin. The technique employed in preparing a slide is often very important in determining what will be basophilic and what acidophilic in reaction. In this respect the fixative is of greatest importance. For example, it is quite improbable that the nuclear membrane is chromatic, and yet it reacts as a basophilic structure after osmic fixation. Furthermore the acceptance of the basophilic point of view would require the belief that the chromosomes and the material from which they arise during division is largely non-chromatic, and that the endosomes, having no relationship to genetic factors insofar as can be found by analogy with other cytological observations are the most chromatic elements of the nucleus.

Bělař's point of view avoids some of the more obvious difficulties. By identifying the chromatin as those materials which are genetically associated with the chromosomes, he develops a functional point of view which makes the actual chemical nature of the material unimportant. To a great extent, this is an interpretation which eliminates all of the objections of the tests mentioned above. If in some organisms, like *E. blattae*, and some of the other Protozoa, as mentioned by Sassuchin

(1936) which show a positive reaction to the Feulgen test at restricted points in their life cycle, there is a chemical change in the material associated with the chromosomes, Bělař's concept of chromatin will include them readily enough.

On one point, however, the interpretation of chromatin as stated by Bělař fails to wholly satisfy the conditions observed in *E. blattae*. It seems logical that if the term chromatin is to have a functional significance as he suggested, it should include not only that material which is most active during the division of the nucleus in distributing genetic factors, but that the active material of the interphase period should also be included. Since the mere distribution of genetic factors is unimportant unless they do function during the interphase condition, the functional interpretation should include the structures which appear to undergo the greatest activity during the interphase. In most nuclei there is no morphogenetic difference between the structures which are derived from the chromosomes and the structures functioning during the metabolic activities of the nucleus in interphase, insofar as we are aware at the present time. In *E. blattae*, however, activity in the interphase appears to center in the endosomes, which, insofar as can be determined, are not derived from the chromosomes at each division and receive no material from the chromosomes during interphase. This leads to the point of view that the endosomes, as well as the central region material associated with the chromosomes, should be considered as chromatic. Since the inner light-staining material seems to be developed during the interphase period, and since it is possibly developed from the outer basophilic portion of the endosomes, the basophilic material is believed to be the active part of the endosomes.

The results of the study of the possible chemical composition of the nuclear elements show that the endosomal material and the chromosomal material are the only elements which appear to be composed of nucleic acid or nucleoprotein at the present time. This functional point of view as outlined above does not diverge an excessive amount from a chemical view, although it seems quite true that the nucleoproteins composing the central region and the endosomes, from their staining reactions, are probably chemically different, or, at least, in a different physical state.

The closest approximation to a specific chromatin test, if the above point of view be maintained, was obtained with the pepsin-hydrochloric acid. Realizing that future work may greatly alter our present concepts, it is impossible to offer any static and final concept, but, with the present information, it seems possible to infer that pepsin-hydrochloric acid digestion does isolate and differentiate between the chromatic and non-chromatic material of the nucleus, and, further, offers an opportunity

to differentiate between the morphological structures which appear to be most active in the interphase and in the dividing stages of the nucleus, at least for *E. blattae*.

XIV. THE PRECYSTIC PERIOD

THE PRECYSTIC organisms may be distinguished from the trophic organisms by their smaller size, their clearer cytoplasm, and the presence of two or more nuclei. They are typically quite active until just before the formation of the cyst wall, when they become quiescent and round up into a spherical shape.

Bütschli (1878) was the first to describe the precystic amoebae. He noticed that in addition to large uninucleate individuals there were a number of smaller organisms with several nuclei. Nuclear size was inversely proportional to the number of nuclei, and the organisms with more nuclei were usually somewhat smaller. The ones with many nuclei were frequently quiescent and rounded in shape. Nuclei were typically spherical, but sometimes were irregularly elongated or spindle-shaped. The appearance of the nucleus differed from that of the larger forms in that the interior was a large fluid-filled cavity, and nuclear contents were restricted to a narrow band at the edge of the nucleus in contact with the nuclear membrane. Bütschli's observations were made on living amoebae.

Schubotz (1905) saw amoebae with up to 20 nuclei. They were regularly smaller than the trophic forms, and he believed them to be precystic. In the multinucleate amoebae the nuclear membrane was much thinner, appearing as a single line instead of a double-contoured structure. In the living condition they were quite similar to the nuclei of the trophic stages, except that there was a smaller number of peripheral granules and nucleoli.

Elmassian (1909) described the formation of two kinds of cysts. He termed these dark and light cysts. The precystic stage of the dark cyst was an amoeba with a single large typical nucleus, with endosomes. It was indistinguishable from the trophic amoeba, except for its clearer cytoplasm and somewhat smaller size. As the organisms secreted a gelatinous coat the nucleus exploded, releasing secondary nuclei, which developed into the cyst nuclei. The light cysts were developed from amoebae containing a single large nucleus which had undergone a morphological transformation involving the total destruction of the endosomes and the formation of a large number of tiny chromatic granules. The nucleus divided and a gelatinous cyst wall was formed, which ended the precystic development.

Mercier (1910) believed that the first step towards encystment was

extrusion of chromatin from the trophic nucleus, after which the nucleus divided. The peripheral and central zones of the two nuclei resulting from this division were poorly defined. During the division of these nuclei a centrosome occurred at each pole, and extending between them was an achromatic spindle. The chromatin occurring on the spindle was in the form of small spherical chromosomes. These divisions were not always synchronous, resulting in the production of amoebae with 3, 4, 5, 6, 7, or 8 nuclei. In the interphase the central region contained an area of achromatic ground-plasm in which large chromatic chromosomes and small achromatic granules could be found. The peripheral zone was finely granular. Nuclear division was initiated by division of a small granule found in the central region, which he termed the centrosome. The finely granular central zone divided, and a spindle extending from pole to pole was formed. The chromosomes became arranged on the spindle and moved to the two poles. The number of chromosomes was not determined in dividing forms, but appeared to be about 8 in the interphase nucleus. At the time of the formation of the cyst wall the nuclei were invariably clumped at the center of the cyst. This occurred when there were 8 nuclei in the amoeba in most cases.

Although Kudo (1926) did not study the precystic stage specifically he described an interesting migration of nuclei in an amoeba apparently preparing to encyst. There were 4 nuclei which moved to the periphery, and at one time a superficial resemblance to budding resulted. Later they were drawn back into the cytoplasm, however.

Morris (1936) found that precystic amoebae were characterized by nuclei which had a reduced amount of peripheral chromatin. The "karyosome" or central granule lying in the central region was much easier to observe in the precystic stage. Nuclear division of a type essentially like that of the trophic nucleus continued until 8 or 16 nuclei were formed. After these divisions a reorganization of the nucleus occurred during which the nuclear wall was reduced in thickness and the chromatin was arranged along the nuclear wall at regular intervals. A number of achromatic radii connected the central region to the nuclear wall. After the nuclear transformation the amoebae rounded up and the nuclei migrated to the center of the body, where they were arranged in a compact clump during the formation of the cyst wall.

XV. THE TRANSFORMATION FROM TROPHIC TO PRECYSTIC PERIOD

THE CYCLE of an infection with *E. blattae* is but little known. Cleveland and Saunders (1930) found that infections with *E. histolytica* follow a regular course, involving a gradually accelerated division rate inversely

proportional to a gradual decrease in the size of the amoebae. Morris (1936, p. 234) remarks concerning *E. blattae*, "Where numbers in infections are comparatively small, the individuals are habitually larger and show fewer indications of recent fission, while the approach of encystment is uniformly accompanied by increasing numbers of smaller animals which are usually in some stage of division or show signs of recent completion of this process."

Several questions are raised by a consideration of these statements. Are the trophic amoebae smaller in heavy infections, and if so, do they show signs of a more rapid division rate? Is a high division rate always accompanied by smaller size and encystment, or is it associated with the density of population? Are cysts produced only in heavily infected cockroaches, and if not, does the division rate increase in amoebae approaching encystment?

Cyst production was observed in both light and heavy infections. In either case, the amoebae which, as shown by their smaller size and less abundant food vacuoles, were approaching the precystic condition showed a definite increase in division rate. This increase in frequency of division was apparently independent of the density of the amoebae population of the host. A larger number of precystic individuals were found in the more heavily infected cockroaches, but not necessarily a higher percentage of precystic individuals. The indications of a higher division rate in the trophic forms in heavily infected hosts were not very clearly expressed, and the large trophic forms of heavily and lightly infected hosts appeared to average about the same size. It becomes rather clearly indicated by these points that the rapid divisions leading to the formation of cysts are independent of population factors in the case of *E. blattae*, at least in some cases, but that the transformation from the trophic to the precystic condition is accompanied by a definite increase in division rate independent of the population density. The increase in division rate leads to a change in the nuclear appearance, which makes it possible to recognize which are developing toward the precystic condition.

The early stages of the transition cannot be followed, for the series of rapid divisions which end in the precystic stage are not unlike those of the trophic part of the life cycle. As the cytoplasm becomes less filled with food vacuoles, however, the amoebae begin to decrease in size as a result of the divisions and lack of food and can be distinguished with comparative ease. The nucleus is smaller and the nuclear membrane is thinner, although still heavy enough to form a double-contoured image under oil immersion. The division of these nuclei is still typical until the late kinetophase is reached. When the chromosomes are clumped at the poles of the daughter nuclei the segregation of the peripheral and chromosomal material is very distinct. The chromosomes do not undergo

the rapid clumping which occurs in the division of the trophic individual, and the migration of the peripheral material around the chromosomal mass is delayed (Figs. 59, 60, 61). The segregation of the two nuclear regions is maintained for a longer time, and the chromosomes often form a loose reticulum instead of massing together (Fig. 62). The reticulum formed by the peripheral and endosomal material is much more slow to migrate around the central region and is noticeably smaller in amount in these stages. By the time that several such divisions occur the amount of endosomal and peripheral material is greatly reduced. Interphase reorganization is rather restricted, as the next division begins very soon after the last is completed. These interphase nuclei are characterized by the much smaller proportion of endosomal and peripheral material to central region than is the case with the trophic amoebae. This appears to result from the failure of the nucleus to form new endosomal material and new peripheral ground-plasm as rapidly as the nuclei divide. Since there is some evidence that this synthesis of peripheral material occurs at least partially in the interphase, the shortening of the interphase period is believed to be a possible factor in bringing about this reduction. The restricted amount of food may be another indirectly operating factor.

Living amoebae during this period are characterized by the more dense and clearer cytoplasm. The food material is reduced in amount and there are few or no food vacuoles. They are extremely active, however, undergoing locomotion which, size considered, is much more rapid than is the case of the trophic amoebae. The nucleus is smaller and the peripheral spherules fewer in number. The endosomal material is often more prominent as a result of the reduction in the peripheral spherules. The central region occupies a relatively larger proportion of the nucleus.

Fixed nuclei are characterized by a thin acidophilic membrane within which lies a narrow band of acidophilic peripheral ground-plasm bearing peripheral granules and endosomal spherules staining deeply with haematoxylin and safranin but negative to the Feulgen test. The granules are relatively few in number. The endosomal material is in the form of small spherules, which are smaller in size, although they lose none of their affinity for basic dyes. They no longer appear to be composed of a basophilic shell and a less basophilic core. The central region is usually acidophilic in its reactions to haematoxylin and, to a smaller extent, to safranin. A basophilic centriole may occasionally be demonstrated. In Flemming-safranin preparations the hyaline body is more prominent than in the trophic nucleus. Its division during the early kinetophase, as described by Janicki (1909), has not been observed, although it can be found in comparatively later stages of nuclear reorganization following division than is the case in the ordinary trophic

amoebae. The kinetophase begins when the central region appears to increase in its affinity for basic dyes. The chromosomal material moves away from the nuclear membrane and forms a coarsely reticular, dedifferentiated stage, which is followed by the arrangement of the peripheral spherules and peripheral granules in rows between the poles of the elongating nucleus. The chromosomes migrate to the two poles, without apparently undergoing a metaphase stage, where they form a rosette of strands, which often may be replaced by a loose reticulum of tangled chromosomal strands. The endosomal spherules become arranged on the reticulum formed by the peripheral ground-plasm in the median part of the nucleus, and shortly thereafter the nucleus begins to constrict. In the daughter nuclei which are formed, the isolation of the peripheral and central zone material is more complete than in trophic individuals. As the hyaline body appears at the poles of the daughter nuclei associated with the chromosomal mass, the definitive central zone is formed. The amount of peripheral material is reduced to a small vestige remaining at the pole opposite the chromosomes. The hyaline body becomes somewhat more resistant at this time and may be located after fixations which destroy it in the normal trophic individuals. Glacial acetic acid will preserve the hyaline body of such amoebae, approaching the precystic condition. It appears to be distinctly more basophilic at this time than is the case in the trophic nucleus.

XVI. THE LIVING PRECYSTIC AMOEBA

THE PRECYSTIC stage begins when a suppression of cytoplasmic division after nuclear division brings about a binucleate condition. Nuclear divisions continue until between 8 and 16 nuclei are present, when a nuclear transformation occurs. Following the nuclear transformation a period occurs during which there is active locomotion, but no food is engulfed and no visible alterations in the nuclei occur. This appears to be a comparatively long period, as pointed out by Morris (1936). The rounding up of the organism is followed by the formation of the cyst wall. Nuclear changes accompany the formation of the cyst.

The early precystic period, extending from the binucleate stage to nuclear transformation, involves several nuclear divisions which have not been observed in life. The active amoebae are about 25 to 35 μ in largest diameter. Few food vacuoles are present, and these are almost invariably incompletely digested. The cytoplasm appears to be somewhat more dense than in the trophic amoebae, but this density is not accompanied by any decrease in activity, for the organisms are always actively motile. They usually move in a "limax" form, with but one broad pseudopodium

being formed. Cytoplasmic striation during locomotion is not infrequent. Cytoplasmic inclusions have been studied in some individuals in permanent mounts and with vital dyes. Mitochondria apparently do not undergo any significant alteration. The neutral red-stainable inclusions are present. The granules are present in large numbers (Fig. 74), but the larger spherules are greatly reduced in number. In many precystic amoebae the spherical inclusions are entirely lacking. The granules are osmiophilic, as in the trophic amoebae.

The nuclei are variable in number. They are usually rather small, the size being reduced as the number of nuclei increase. The nuclear membrane is relatively thin, and the transparent central region is relatively much larger than in the trophic amoebae. The number of peripheral spherules is much smaller than in the trophic nucleus. They lie in the peripheral zone, imbedded in the peripheral ground-plasm which is also greatly reduced in amount. Endosomal spherules, somewhat less refractive than the peripheral spherules, are usually more numerous than in the trophic nuclei, and are much smaller.

During nuclear transformation no great alteration in the cytoplasm occurs. The amount of food decreases, and the cytoplasm is usually completely free from food vacuoles by the end of the nuclear transformation. The nuclear appearance alters comparatively little insofar as the living nuclei are concerned. The more refractive peripheral spherules disappear, but are replaced by endosomal spherules. These are usually quite deliberate in their migration around the central region after division, and are frequently seen surrounding half of the nucleus in a "crescentic" area. The alterations in the central region, as seen in fixed nuclei, are apparently not visible in life. The nuclear membrane becomes much thinner.

After transformation of the nuclei into their new structure, the amoebae remain actively motile for a time. This is followed by a rounding up of the organism, during which the cytoplasm becomes noticeably more dense. At this time the larger inclusions stained by neutral red disappear, although granules appear to occur. There are indications of a reduction in the number of the smaller inclusions, also, but this is not so striking as the disappearance of the larger spherical inclusions. The formation of the cyst wall appears to be a rather slow process during which some cytoplasmic streaming occurs. No other visible activities occurred. It may be that it occurs more rapidly in hosts than it does in depression slide mounts, but no means of determining its speed in the host has been devised. On slides it requires from one to several days for completion.

XVII. THE EARLY PRECYSTIC PHENOMENA

THE APPEARANCE of the nuclei in the early precystic amoeba is quite like that of the nuclei seen in the rapidly dividing "precystic" organisms. Each nucleus is smaller after each division, although the ratio of nuclear mass to cytoplasmic mass appears to be increased during the divisions. The nuclear membrane is rather thin, but reacts to stains and fixatives exactly as the nuclear membrane of the trophic amoeba. It is homogeneous after all fixatives, and, in all but osmic fixations, is acidophilic.

The peripheral zone is restricted in extent. There is a much reduced amount of ground-plasm, which is usually homogeneous or finely reticular after fixation. As in the trophic nucleus, fixation with low grades of acetic fails to preserve the ground-plasm. The basophilic peripheral granules are much smaller in number than in the trophic nucleus. They are present in small numbers during the early precystic period, however, although they appear to become less numerous as the number of nuclei increases. There are a few peripheral spherules in most nuclei, visible after alcoholic or formaldehyde fixation. They are resistant to staining.

The endosomal material is present in the form of tiny spherules which are unstained in Feulgen preparations, but are distinctly basophilic. These small spherules, visible in life, are very rarely fused into true endosomes, although this does occasionally occur in early stages. The occasional nuclei in which endosomes are formed from the endosomal spherules never contain elongated strands, but instead the spherules mass together into spherical clumps which rapidly become smoothly rounded (Fig. 65). The endosomes and the endosomal spherules are well preserved by all of the compound fixatives. Although the spherules are usually attached to the nuclear wall they are sometimes clumped around the central region (Fig. 70). They are sometimes irregular and appear to be less basophilic after alcohol fixation. Although the endosomes were rather poorly fixed in trophic amoebae by glacial acetic acid, this is less noticeable in the precystic amoebae. They are well preserved by glacial acetic acid. The visible differentiation of light-staining and dark-staining substances is not found in the precystic endosomal material. The fact that all of the endosomal material appears to be equally well preserved in glacial acetic acid and 10 per cent acetic acid when the light-staining material is absent supports the view that it is the light-staining material which was poorly preserved in the trophic endosomes by acetic acid. The shorter interphase period may account for the failure of the less basophilic material to form, if the basophilic material is indeed broken down in the interphase stage into the less basophilic vacuolar material.

The central region of the early precystic amoeba is but little altered.

Radii connect the central region to the peripheral region, and in some cases the radii appear to penetrate through the peripheral material and attach to the nuclear membrane. The number of radii is not constant in the precystic amoebae, nor in the trophic amoebae, thus contrasting with the constancy in the number of radii in *Entamoeba histolytica*, reported by Kofoed and Swezy (1925). The central region is usually homogeneous, but in some cases may appear somewhat fibrous. The fibrous consistency is seen most frequently in material fixed in chromic acid or Zenker's fluid. The central region is usually acidophilic, but following a chromic acid or Zenker fixation, it is quite basophilic. This appears to be the result of a mordanting action of the chromic acid rather than the presence of basophilic material not fixed by other reagents. The actual amount of central region material appears to decrease during the several nuclear divisions, but much less than the peripheral material. It is little more conspicuous than in the trophic amoebae, in spite of the smaller amount of obscuring peripheral material. The hyaline body can be found after appropriate fixation. It is more prominent than in trophic nuclei, and is somewhat more resistant to deleterious fixations. Although it cannot be traced throughout the whole interphase, it appears to last during a greater portion of the interphase than is the case in the trophic nuclei.

Early precystic divisions are typically like the trophic divisions. The first division producing four nuclei is rarely exactly synchronous, so that a trinucleate stage is rather common. In these trinucleate forms one nucleus is usually in the early kinetophase condition, while the other two are in the early interphase. The first division seems to be somewhat atypical, especially in some amoebae. Mercier (1910) described a clumping of chromatic constituents together in the binucleate stage to form a transitory karyosome. The further changes of this structure are described as follows (p. 158), "Le caryosome se décompose et donne d'une part les nucleolés et d'autre part un appareil centrosomien (centrosome et sphere) qui est situé dans la zone claire du noyau." This karyosome described by Mercier has not been found, unless he refers to the hyaline body. Occasionally a large endosome formed from several endosomal spherules occurs, but in no case can this be considered as similar to the karyosome mentioned by Mercier. Mercier figures (Fig. 24) a division from the binucleate to the trinucleate stage. He finds a centrosomal granule at each pole and a number of chromatic bodies which extend between the granules. In the material of the present investigation these atypical divisions have occasionally been observed. The normal division figures are characterized by their comparatively round shape and bluntly rounded poles (Fig. 67). No chromosomes could be found in these nuclei. These may have represented nuclei in the so-called "dedifferen-

tiated" stage which were beginning to elongate. In other cases, nuclei dividing in a typical fashion were found.

The tetranucleate organism is capable of undergoing nuclear transformation, but this is apparently a rare occurrence, the change in nuclear structure usually occurring in the 8-nucleated stage. Since the divisions are not wholly synchronous, the number of nuclei do not coincide with the powers of two. In rare cases a full 16 nuclei are formed before transformation occurs.

The precystic divisions following the first one are typical. Endosomal spherules lie in the median part of the elongated nucleus, and the chromosomes are found at the poles (Fig. 68), where they are sometimes clumped in a slightly basophilic mass. In the constricted daughter nuclei, the typical telephase appearance is usually observed (Fig. 69). There are some indications that a reduction in chromosomal material has occurred, for the chromosomal clump is noticeably smaller in size, and attempts to count the chromosomes give consistently lower numbers. Until the number of chromosomes has been definitely determined in the trophic stage, however, no definite meiotic processes can be recognized.

Constriction of the nucleus leads to the late kinetophase reconstruction. The basophilic endosomal spherules, imbedded in the peripheral ground-plasm, lie in a crescentic mass at one pole of the daughter nucleus after the nucleus has rounded up (Fig. 73). At the opposite pole are the chromosomal strands which are slightly basophilic in haematoxylin preparations, but are very clearly basophilic in safranin preparations. The chromosomes are positive to the Feulgen test. The hyaline body may be found at the pole of the dedifferentiating chromosomes, but it is smaller than in the trophic nucleus. The karyolymph sometimes forms a spherical mass between the chromosomes and the peripheral material.

XVIII. NUCLEAR TRANSFORMATION

THE END of the early precystic development during which the increase in number of nuclei was accomplished is marked by a complete alteration in nuclear morphology. The nuclear transformation appears to begin with a gradual increase in the nucleic acid content of the central region, as indicated by the Feulgen reaction. At first the central region is stained a diffuse violet color in Feulgen preparations, but the shade gradually becomes more intense as the transformation of the remaining components advances. It appears that the peripheral spherules disappear at this time, and it is suggested that if they are truly a phosphoglobulin-like substance, they may supply the central region with a phosphorous compound utilized in producing nucleic acid. At one pole of the central region a

small basophilic granule appears. Its position is such that it seems to have been derived from the hyaline body, but it is entirely unlike the hyaline body in its reactions to stains and fixatives. It is clearly distinct from the central granule found in the trophic and precystic nucleus, for this nuclear element can be seen in the center of the central region at the same time that the larger eccentric granule is present (Fig. 71). Morris (1936) says that the "karyosome" or centriole is more conspicuous in the precystic amoeba than in the trophic form. This does not seem to be the case, for the centriole is quite difficult to demonstrate in the precystic amoebae. Morris may possibly have mistaken the eccentric body for the central granule or centriole. The eccentric granule increases in size (Fig. 72) and becomes still more noticeably positive to the Feulgen test at this time. The peripheral material is not greatly altered. The ground-plasm is slowly decreasing in amount and the basophilic endosomes and endosomal material are concentrated into smaller granules which finally become arranged along the inner surface of the nuclear membrane at relatively regular intervals. This occurs at different speeds, so that no definite time for the completion of the redistribution of the peripheral material can be specified. It is usually completed before the central region alterations are completed, but this is not invariably the case.

The eccentric basophilic body becomes altered by its disruption into several distinct granules during later development. This seems to differ somewhat in different fixatives, but the reason for this variation is not wholly understood. With strong alcoholic fixatives the body generally forms a small basophilic circlet (Fig. 76), which is at first undifferentiated and homogeneous along its whole length, but later forms four or five distinct granules, connected by more lightly-staining material (Fig. 77). The circlet is less prominent after Zenker fixation, but usually can be found in favorable cases. In osmic-containing fixatives, however, the circlet is almost invariably lacking. In its place there is a larger eccentric body, from which the four or five granules are formed directly.

The appearance after transformation approaches the morphology of the cyst nuclei, but is very unlike that of the trophic nucleus. The nuclear membrane is comparatively thin. Along its inner surface there are a number of small basophilic granules developed from the endosomal spherules, which are arranged at almost regular intervals. They are negative to the Feulgen test, and have been derived from the peripheral material of the trophic nucleus. The central ground-plasm is acidophilic, and contains a small central granule which is quite inconspicuous, although basophilic. The eccentric basophilic circlet and associated granules are positive to the Feulgen test, and after the completion of the nuclear transformation appear to contain the majority of the nucleic acid found in the nucleus.

XIX. THE LATE PRECYSTIC PHENOMENA

THE NUCLEI may divide after the nuclear transformation, before the formation of the cyst wall, but this does not appear to occur in all cases. This division, when it does occur, is atypical. It is initiated by a transverse division of the basophilic, eccentric circlet (Figs. 77, 78). After division of the circlet, the two halves each unite to form a smaller circlet (Fig. 80) which migrate to opposite poles of the central region. The next step has not been observed, but apparently consists of an elongation of the nucleus and the migration of the daughter circlet to the pole of the nucleus. Once arrived at the poles, the circlets remain in this position, while the elongated nucleus constricts (Fig. 81), forming two daughter nuclei. Chromosomes have never been seen in this type of division. The rarity of the dividing figures suggests that division may be very rapidly completed, or that it is quite rare. It occurs most frequently in precystic amoebae with few nuclei. It is probable that, since nuclei have been seen in the early division stages more frequently than in the later stages, the actual elongation and constriction of the nucleus occurs in a comparatively short space of time. Reconstruction after division leaves the nucleus with a small circlet, about half the size of the normal circlet (Fig. 80).

The nuclei undergo another morphological transition as the cyst wall formation approaches, and the amoeba rounds up and becomes inactive. The cytoplasm acquires an odd striated appearance which has been described by Mercier (1910) and Morris (1936). These investigators also observed that the nuclei migrate to the center of the organism where they form a small clump. The nuclear structure is wholly altered. The interior of the nucleus is filled with a spongy reticulum which bears a large number of granules. In its general appearance it is quite similar to the dedifferentiated stage of the dividing trophic nuclei, from which it differs primarily in that the particles occurring on the spongy net are deeply basophilic and positive to the Feulgen test (Fig. 82). As the nuclei acquire irregular shapes the granules which occur on the reticulum become quite darkly colored by the Feulgen reaction, and the nucleic acid content appears to reach its peak. At no other time during the life cycle, from trophic amoeba to mature cyst, does there appear to be such a high concentration of the nucleic acid. During the succeeding activities the nucleic acid content is rapidly reduced, and it is believed that the nuclei are active in some way during the formation of the cyst wall. Their irregular shape, and their indistinct outlines appear to support this view. Later changes, to be described with the cystic phenomena, suggest that these changes may be considered as early kinetophase activities in part.

XX. THE CYSTIC PERIOD; HISTORICAL REVIEW

WHEN THE deposition of the cyst wall occurring at the end of the pre-cystic period is completed, the amoebae pass into the cystic stage. The wall is thick and hyaline, and quite conspicuous. At this time they begin to pass down to lower parts of the alimentary tract.

Bütschli (1878) was the first to describe the cysts. He mentions thick-walled multinucleate cysts which were probably a part of the life cycle of the amoeba. He noted that the nuclei were much smaller in the cysts, ranging from about 3 to 8 μ .

Schubotz (1905) described the cyst nuclei as varying from about 4 to 6 μ . Each cyst contained from 20 to 30 nuclei. The nuclei were spherical to elliptical, and the central region was much less prominent than in the trophic forms. The cyst nuclei appeared to be finely granular, and few refractive granules occurred along the nuclear membrane. The nuclei usually lay in the hyaline part of the cytoplasm, which became divided into two zones, a finely granular darker part and a hyaline lighter part. He noted that in some cysts nuclei were found lying side by side, with adjacent parts of the nuclear membrane flattened. He believed that this might represent division or copulation. Since there was no reduction in number of nuclei in the older cysts, Schubotz came to the conclusion that these paired nuclei were more probably dividing than copulating. A week in a moist chamber did not bring about any alterations, but cysts from faeces which had been dried showed distinctive differences. The cyst wall, while as thick as in the fresh cysts, was often irregular. The cytoplasm was uniformly granular throughout, as the hyaline protoplasm had disappeared. The nuclei were irregularly distributed over the whole cyst, but differed little or none from the nuclei in the fresh cysts. Although he was unable to bring about development from cysts in feeding experiments he observed a number of very small amoebae (6 to 8 μ) which were characterized by their hyaline cytoplasm and active movements. He believed that these were young *E. blattae*.

Elmassian (1909) studied encystment in the colon of the host and in material forced to encyst by leaving the trophic amoebae in solutions of salt containing the teased apart host colon at 12° C. and at room temperature. At room temperature encystment was rather capricious, but at low temperatures the course of the encystment was slow and many amoebae were still motile after 9 days in salt solution. He found two kinds of cysts, light and dark. The cyst wall was laid down in the uninucleate stage in the case of the dark cyst. Secondary nuclei developed into the cyst nuclei. At the time of their release into the cytoplasm, the secondary nuclei were nearing division, and they formed a primitive spindle on which fine chromatic granules were distributed. The bursting

of the nucleus left a fine chromidial residue, which formed a reticulum throughout the cytoplasm. The secondary nuclei underwent several divisions until their number was considerably increased. In some of the nuclei a halo of chromatic material lay around them which was at first crescentic in shape and then formed distinct granules. He believed that this was a process of chromatin diminution. At this time the nuclei often appeared to be in pairs, which he described as a fusion of gametic nuclei. The contents of the cyst assumed a new form at this time. The nuclei divided, increasing in numbers, but in doing so, decreasing in size, while the cytoplasm separated into light and dark plasms. The granular dark plasm was differentiated from the hyaline light plasm by its affinity for basic dyes, which he considered to be due to the remains of the chromidial net. The cytoplasmic differentiation was lost by the time that the cysts were expelled from the colon. As many as 72 nuclei were found in one cyst. Small chromatic granules occurred at the periphery of the small nuclei found in the ripe cyst. Elmassian believed that there were two divisions involved in cyst formation, the first of which was a true mitosis.

The clear cysts had fewer nuclei. As described in the precystic discussion, they were derived from a binucleate precystic form, which secreted a cyst wall. The two nuclei were characterized by a very thin nuclear membrane enclosing a fine reticulum on which there were a large number of fine chromatic granules. The two nuclei prepared to divide, elongating and assuming a characteristic spindle shape. A chromatic mass occurred at each pole of the elongated nuclei. The two spindles constricted in the center, producing four spindles, which changed shape somewhat, and then divided into 8 nuclei. Two more divisions took place, producing 32 nuclei in the mature light cysts. The spindles observed during the divisions involved in the cyst development were characterized by small granular chromosomes. There was no centriole. The irregular chromatin occurring at the poles of these spindles, Elmassian believed to be something other than chromosomes.

Elmassian was of the opinion that the light cysts represented a schizogonic cycle, while the dark cysts, in which the paired nuclei fuse, represented a sporogony, similar to that occurring in the Sporozoa.

Janicki (1909) noted that cysts were usually formed from small amoebae with 8 nuclei. Mitotic divisions similar to those found in the trophic amoebae occurred in precystic development, but cyst divisions were different. The karyosome initiated division by elongating and becoming slender. Two centrioles appeared at the poles of the elongated karyosome, which Janicki was inclined to believe originated by the division of a single centriole. No structure connecting the two centrosomes was present. A small amount of indistinct chromatin was

present at that time. Within the nuclear membrane a spindle was formed. An equatorial plate stage was easily found at the time that the nucleus was spindle-shaped. There were six spherical chromosomes which moved to the poles and gathered together in rather indistinct masses. The karyosome reappeared in the resting stage, surrounded by granular chromatin. Up to the stage with 8 or 16 nuclei the divisions were more or less synchronous. Cysts with up to 30 nuclei were found.

Mercier (1910) considered encystment as a process allied with gametogenesis. The cyst stage was reached when the amoebae were in the 8-nucleated stage in most cases. As the cyst wall began to form the nuclei were clumped in the center of the organism. The cytoplasm was distinctly striated at that time, but the striae disappeared very soon after the cyst wall was completed. When the cytoplasm regained its homogeneous condition the 8 nuclei divided more or less synchronously. The divisions were mitotic, but the nuclear membrane persisted through this and succeeding divisions. The later divisions were more difficult to follow because of the small size of the amoebae. Chromidia occurred in the cytoplasm, derived from the precystic reduction process. Cysts so formed were passed out with the faeces.

Morris (1936) was the last to undertake a study of encystment. He observed that the nuclei of the precystic amoebae migrated to the center of the body at the time of cyst wall formation. The cytoplasm became more dense as the cyst wall was secreted. After the cyst wall was formed one nuclear division followed immediately. The small size of the nuclei made it impossible to study the division satisfactorily but he observed that there were indications that it was atypical. After this division no further nuclear changes occurred, and the two cytoplasmic phases separated into a denser, hyaline material and a more fluid granular material occurring in the form of large vacuoles. The ripe cysts passed out of the host in the faeces and infected new cockroaches.

XXI. APPEARANCE OF FRESH CYSTS

THE CYSTS vary in size from about 20 μ to almost 50 μ . They are very easy to recognize because of their refractivity, and may be located readily even under low magnifications. The cyst wall is relatively thick, sometimes attaining a thickness of 4 to 5 μ , but usually measuring 2.5 to 3.5 μ . It is refractive and hyaline, and is closely pressed against the cytoplasm lying within it. The outer surface of the cyst wall is relatively smooth in fresh cysts, but, as pointed out by Schubotz (1905), it becomes quite irregular in cysts which have been dried in faeces. The differentiation of the hyaline and granular plasma is quite difficult in living cysts but can

be made out with careful study. The nuclei are quite small, and in many cysts are invisible in life. The smallness of the nuclei, and the fact that their refractive index approaches that of the cytoplasm makes it impossible to study them satisfactorily in life.

XXII. STUDY OF FIXED AND STAINED CYSTS

THE FIXED and stained cysts undergo some shrinkage during treatment, but fall in the same size range as the living ones. The cytoplasm is frequently somewhat basophilic, and it is extremely difficult to accomplish satisfactory differentiation.

The cyst wall is quite heavy and shows indications of stratification in many cases, especially after sectioning. The cyst wall is quite resistant to staining with basic dyes, and is lightly stained or unstained with the cytoplasmic dyes. The cytoplasm at the time the cyst wall is formed is striated. As described by Morris, the cytoplasm becomes homogeneous after the deposition of the cyst wall. At this time it is hyaline and appears to be quite dense. During later development vacuoles of a granular, more basophilic plasma appear in it. These seem to appear near the periphery first, and fuse together, later migrating to the center of the cyst. The mature cyst usually contains from one-half to one-third granular plasma. In older cysts this may be even more pronounced, and Schubotz (1905) found that in week-old dried cysts all of the cytoplasm is granular. This suggests that Elmassian's view that the granular part of the cytoplasm represents chromidia is very doubtful. Another viewpoint is expressed by Morris (1936, p. 235): "At the close of the cystic nuclear division, the two cytoplasmic phases, described earlier in the active adult amoeba, separate from each other; the denser, less fluid portion forming a continuous matrix wherein the nuclei rest, while more fluid material forms a series of large vacuoles at the periphery of the cyst." It seems much more probable, in view of the fact that the whole of the cytoplasm is at first homogeneous, and gradually becomes divided into a hyaline and a granular type of plasma, that the two were not present at first as two distinctly different types of plasma. The writer is inclined to believe that there is a gradual formation of the granular from the hyaline plasma, which may, possibly, result from certain metabolic activities which occur. At any rate, the hyaline plasma may be entirely converted into granular plasma after a period of drying. As observed by all previous investigators who have studied the cysts, the cyst nuclei appear to prefer the hyaline plasma.

During the late stages in the cyst wall formation the nuclei are irregular and somewhat elongate. In a few cases the nuclei begin to become

regular in outline while the cyst wall is quite thin, but this does not appear to be general. After the cyst wall is completely formed, the nuclei regain their regular outline, and appear as somewhat elongated, usually bluntly pointed nuclei (Fig. 83). The thin acidophilic nuclear membrane contains an acidophilic ground-plasm, which in some cases appears to have formed a spindle. In no case have spindle fibres been seen, however, and if the spindle occurs it is quite indefinite. Arranged in a single girdle around the center of the nucleus lie a number of granules which are intensely basophilic and react well to the Feulgen test. These granules are arranged in rows parallel to the long axis of the nucleus. Although they are not exactly like the chromosomes in the trophic nucleus in their derivation or in their appearance, their obvious relation to division processes has induced the writer to apply the term chromosome to them. They divide into two groups which move toward the poles. Whether this is accompanied by a splitting of each granule has not been determined. During the migration period the rows of granules are irregular and become indistinguishable. Janicki's (1909) metaphase of the cyst nuclei probably corresponds to these atypical spindles and chromosomal granules. The spindle is elongated during the migration of the granules to the poles, and a constriction divides the spindle into two daughter nuclei. Division of spindles into daughter spindles, as described by Elmassian (1909) has not been observed.

The cyst nuclei arising from these spindles are characteristic in shape and structure. They are spherical and rather small, averaging between 4 and 5 μ in diameter. The nuclear membrane is quite thin, and within it lies an area of basophilic material which is in contact with the membrane along its whole periphery. In some cases this is irregular in distribution, forming a crescentic mass at one side of the nucleus. This seems to be identical with the "halo" mentioned by Mercier (1910) and Elmassian (1909). Insofar as could be determined the nucleus does not extrude chromatin into the cytoplasm. Within the basophilic area there are usually several discrete chromatic granules which appear to be derived from the endosomal granules of the precystic nucleus (Fig. 90). Within the peripheral basophilic zone there is a narrow light-staining region containing a small basophilic granule in the center of it (Fig. 90). This is apparently identical with the centriole observed in the trophic nuclei.

Two other types of nuclear division were found. In several cysts a division typically like the trophic divisions was found. In these nuclei the chromosomes are apparently quite similar in nature to those found in the trophic form. They migrate to the poles of the elongated nucleus, and between the two clumps of chromosomes there is an area of dark-staining granules (Fig. 84). The question as to whether the division figure repre-

sents a second cystic division will be discussed later. The chromosomes found at the poles of such dividing nuclei are more basophilic than the trophic ones. Definite basophilic granules stained with haematoxylin occur in them. Like the first division, it is synchronous.

There is a third type of division figure which is quite rare. It was observed in two small cysts. The nuclei were much elongated and had begun to constrict in the median line. There were vague signs of a spindle, as in the division figures first mentioned in this section, but no visible spindle fibres occurred. Basophilic structures in these nuclei were limited to four granules at the poles of the nuclei, which were almost exactly like the atypical precystic division (Figs. 85, 86). Chromosomes and endosomal spherules appeared to be wholly absent. The possible significance of this type of division is not known.

The question as to whether there is more than one division in cystic development has not yet been definitely settled. It appears certain that there is more than one division, for although but few of the precystic amoebae have more than 16 nuclei in them, and most contain about 8 nuclei, the cysts almost invariably contain at least 32 nuclei, and may have as many as 72. Morris (1936) says that the precystic amoebae contain from 4 to 16 nuclei and that the cysts may contain from 64 to 72 nuclei. As to the methods of increase of nuclei after the first cystic division, Morris leaves us in doubt, and although a single nuclear division will not suffice to increase the nuclei from the average precystic to the average cystic numbers, he says (p. 235), "A final nuclear division immediately follows encystment," and again "Mercier believed that more than one postencystment division might occur, but the present observations do not confirm this view." It seems quite possible that the first nuclear division is followed by the type of division described as similar to the trophic divisions. It was interesting to notice that although the chromosomes could not be accurately counted because of their small size and the poor differentiation which was frequently obtained, there seems to be more than half as many as in trophic divisions. This may indicate that no meiotic phenomena have occurred, or that nuclear fusion occurs in the cyst as believed by Elmassian. No evidence on either point is at hand.

Some evidence for an amitotic division of cyst nuclei has been discovered. In several cysts a few nuclei were found in which the nuclei were pear-shaped and appeared to have recently constricted (Fig. 88). There were no signs of chromosomes or spindle, and it resembled an amitotic division in all respects. Variable size among the cystic nuclei has been observed in many cases, suggesting that there may be occasional nuclear divisions following the synchronous mitotic ones (Fig. 87).

At the time of cyst wall formation the nucleic acid content of the cystic nucleus is very high. During the first division of the cystic nucleus the granules lying on the spindle are colored a deep violet red by the Feulgen test. The second type of division is also a period when the amount of nucleic acid is quite high. After this second division, however, the nuclei gradually become less deeply colored, and by the time that the cyst has reached maturity the nuclei are almost completely negative to the Feulgen test.

XXIII. SUMMARY

(1) THE CYTOPLASM of *Endamoeba blattae* is differentiated into ectoplasm and endoplasm. The relative amount of the former is usually greater in less active organisms. The endoplasmic streaming is fountain-like in active amoebae, the central axial stream breaking anteriorly into superficial currents which stream back beneath the surface. In at least some amoebae there appears to be a gelation about one-third to one-fourth of the way back from the anterior end, and a posterior region where solation occurs. When the organism is among detritus, and in other cases, gelation and solation may not occur. Other types of pseudopodial formation occur and are discussed briefly.

(2) The cytoplasm contains a number of small thread-like mitochondria which may be related to the food vacuoles. These are not noticeably altered during the development of the precystic amoebae.

(3) Inclusions which are osmiophilic and argentophilic occur and are stained by neutral red. They fall into two groups, one larger type consisting of a chromophobic core and a chromophilic cortex. The cortical portion, when vitally stained, consists of a number of small granules, apparently identical with the second type of inclusion occurring in the cytoplasm; small, intensely chromophilic granules, almost uniform in size. The spherules are sometimes in contact with the food vacuole wall. The function of these inclusions is discussed briefly, without drawing any definite conclusions. The spherules are reduced in number in the precystic amoebae, although the granules are apparently not affected.

(4) The trophic interphase nucleus is a large heavy-walled structure with a characteristic concentric arrangement of parts, involving a peripheral zone and a central region. In living nuclei the peripheral region consists of a homogeneous ground-plasm enclosing larger peripheral spherules which are highly refractive, and smaller less refractive granules. In fixed nuclei the peripheral ground-plasm appears as a finely reticular or homogeneous region which contains smaller basophilic granules and larger spherules which are resistant to staining with acid and basic dyes. At the inner margin of the peripheral zone there are a variable number of

endosomes composed of a basophilic outer and a lightly-staining inner region, containing a small basophilic central granule. The acidophilic central mass is transparent in living nuclei and is usually reticular, but may be homogeneous, in fixed nuclei. At the center of this central region a basophilic centriole is found in many nuclei.

(5) During division the central region becomes more basophilic and a small amount of nucleic acid, as determined by the Feulgen nuclear reaction, appears in it. At this time the centriole is frequently double. The nucleic acid content of the central region increases as the chromosomes appear. The chromosomes are beaded strands which shorten and become condensed into homogeneous strands which have a high nucleic acid content. Just before the chromosomes migrate to the poles the endosomes are broken down and the remaining basophilic material forms small basophilic endosomal spherules. Some of the peripheral granules may also form similar endosomal spherules. The concentric arrangement is disrupted as the endosomal spherules migrate to the center of the elongating nucleus, where they become arranged in longitudinal rows. The chromosomes migrate to the poles and form a rosette, after which the nucleus constricts and forms two daughter nuclei. The chromosomes mass together as they dedifferentiate and form a new central region which rapidly loses its nucleic acid and affinity for basic dyes. The chromosomal dedifferentiation is associated with the appearance of a hyaline body which is at first basophilic, but gradually becomes less basophilic after the new central region is formed, finally disappearing during the middle interphase.

(6) The interphase nucleus undergoes an alteration with respect to endosomal appearance, thought to be associated with metabolic activities. The early elongated endosomal anlage are formed from the endosomal spherules and a light-staining substance, the derivation of which is unknown, at the time that the chromosomes dedifferentiate. They migrate around the central region with the peripheral ground-plasm to renew the concentric arrangement of the nucleus. The early beaded appearance is supplanted by a homogeneous condition, and the strands first become sinuous, and then shorten and become straighter, finally assuming a cuboidal or spherical shape. After the spherical shape is acquired the endosomes differentiate into a basophilic cortex and light-staining inner region.

(7) The nuclear membrane is composed of a protein substance which is apparently in the form of a rather concentrated colloid, possibly mixed with a non-protein substance. The chemical nature of the nuclear elements is quite uncertain, but an attempt to throw some light on their composition is made.

(8) A series of specific chromatin tests were applied to the nucleus with varied results. It is suggested that chromatin may be considered as the active material during the interphase and kinetophase phenomena, which would be the chromosomal material of the central region and the outer basophilic part of the endosomes. This is isolated from the remaining nuclear material by pepsin-hydrochloric acid digestion, and probably represents all of the nucleic acid and nucleoprotein found in the nucleus.

(9) A series of rapid divisions of the trophic amoeba leads to the development of precystic amoebae. The divisions are typical and result in a decrease in the quantity of peripheral ground-plasm and in the number of peripheral spherules and peripheral granules. The endosomal material usually remains in the form of small spherules instead of uniting to form true endosomes.

(10) The early precystic development includes several divisions which may be more or less synchronous, and which end in a nuclear transformation. From 4 to 16 nuclei may be present at transformation, but there are usually from 8 to 12. The early precystic divisions are quite like the trophic divisions, the only important difference being the clear appearance of a basophilic granule at each pole of the division figures. It is thought that this may be identical with the centriole, but exact information is lacking.

(11) Nuclear transformation follows the early divisions. It involves the appearance of a basophilic body at one pole of the central region, which develops into four or five granules after passing through a circlet stage. The four granules are arranged in the shape of a circle, and are usually connected by areas of more lightly-staining substance. The peripheral material disappears except for a few small endosomal spherules and a very small amount of peripheral ground-plasm. The endosomal spherules become distributed over the nuclear membrane at more or less regular intervals, where they remain. As the peripheral spherules disappear the nucleic acid content of the central region increases. This may be associated with the disintegration of the phospho-globulin thought to compose the spherules. The nucleic acid is at first distributed diffusely throughout the central region, but finally becomes concentrated in the circlet of granules.

(12) Division after nuclear transformation may occur but does not seem to be common. When it does occur it is atypical and no chromosomes can be found. Division of the circlet initiates total division, and the daughter circlets at the poles of the elongated nucleus are the only basophilic parts of the division figure.

(13) The nuclei migrate to the center of the amoeba at the time of

the formation of the cyst wall, when the organism becomes rounded and the cytoplasm assumes a characteristic striated appearance. The nuclear structure is broken down into a coarse reticulum on which a number of chromatic granules occur. At this time the nucleic acid content reaches its peak, and the nuclei appear to be affected by the secretion of the cyst wall, and become quite irregular.

(14) The nuclei regain their regular outline after the cyst wall is formed and develop directly into division figures containing a poorly differentiated spindle on which small granules are arranged in rows. These move toward the poles, where they form an indistinguishable mass and develop into cyst nuclei. During this division the nuclei are quite positive to the Feulgen test, and the cytoplasm appears homogeneous.

(15) Two other types of division figures are described from cysts. Both are synchronous divisions, and one is like the early precystic divisions, while the other resembles the division of the precystic amoebae after nuclear transformation. The former type, which occurs more frequently, is considered as a possible second cystic nuclear division.

(16) The mature cysts gradually become less homogeneous, and develop a granular cytoplasm. The nuclei are very small, with a narrow band of basophilic material along the nuclear membrane, and a comparatively large, light-staining central region. The nucleic acid rapidly diminishes in amount after the last nuclear division, and very little is present in mature cysts.

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PLATES

NOTE: All figures are made from camera lucida drawings, and are magnified 1450 times. Leitz oil immersion 1/12; ocular 4.

PLATE I

- FIG. 1. Late kinetophase. Endosomal anlage migrating around developing central chromosomal mass. Gilson-Carnoy, haematoxylin, orange G.
- FIG. 2. Late kinetophase. Slightly later stage than Fig. 1. Gilson-Carnoy, haematoxylin, orange G.
- FIG. 3. Early interphase. Continuous peripheral region; endosomal anlage becoming homogeneous. Gilson-Carnoy, haematoxylin, orange G.
- FIG. 4. Early interphase. Hyaline body at pole of developing central region. Flemming, safranin.
- FIG. 5. Early interphase. Destained more than usual. Note rows of endosomal spherules and absence of peripheral granules. Gilson-Carnoy, haematoxylin.
- FIG. 6. Early interphase. Endosomal material not formed in straight lines. Gilson-Carnoy, haematoxylin.
- FIG. 7. Middle interphase. Endosomes in the form of sinuous strands. Surface view of nucleus. Gilson-Carnoy, haematoxylin.
- FIG. 8. Middle interphase. Endosomes contracting. Gilson-Carnoy, haematoxylin.
- FIG. 9. Middle interphase. Endosomal spherules still visible, due to transparency of stain. Hyaline body becoming less basophilic. Flemming, safranin.
- FIG. 10. Middle interphase. Sinuous endosomes. Flemming, haematoxylin.
- FIG. 11. Middle interphase. Endosomes gathering at margin of the peripheral zone. Gilson-Carnoy, haematoxylin.
- FIG. 12. Middle interphase. Sinuous endosomes, homogeneous and at their definitive position. Hyaline body gone. Flemming, safranin.
- FIG. 13. Middle interphase. Endosomes oriented in parallel direction. Gilson-Carnoy, haematoxylin.
- FIG. 14. Late interphase. Differentiated less than usual. Note the granular peripheral region and irregular endosomes. Gilson-Carnoy, haematoxylin.
- FIG. 15. Late interphase. Endosomes spherical; hyaline body still present. Flemming, safranin.
- FIG. 16. Late interphase. Endosomes cuboidal. Gilson-Carnoy, haematoxylin.
- FIG. 17. Late interphase. Endosomes spherical; hyaline body not present. Flemming, safranin.
- FIG. 18. Late interphase. Endosomes spherical, showing division into two types of substance. Carnoy, haematoxylin.



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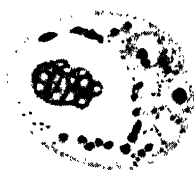
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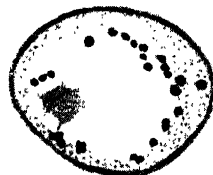
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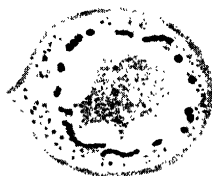
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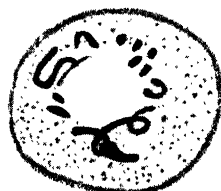
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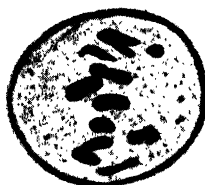
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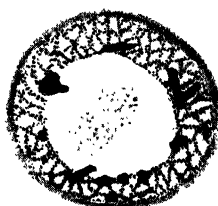
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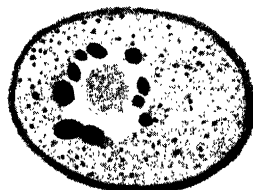
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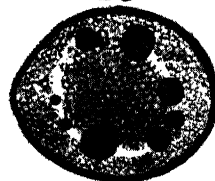
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PLATE II

- FIG. 19. Late interphase. Note poor preservation of endosomes. Carnoy, haematoxylin.
- FIG. 20. Late interphase. Schaudinn, haematoxylin.
- FIG. 21. Early kinetophase. Chromosomal strands appearing in the central region; centriole double. Gilson-Carnoy, haematoxylin.
- FIG. 22. Early kinetophase. The "dedifferentiated stage." Endosomes breaking down to form endosomal spherules. Gilson-Carnoy, haematoxylin.
- FIG. 23. Early kinetophase. Later stages of endosomal disruption. Gilson-Carnoy, haematoxylin.
- FIG. 24. Middle interphase. Endosomal spherules median; chromosomes appearing at edges of girdle of endosomal material. Gilson-Carnoy, haematoxylin.
- FIG. 25. Middle interphase. Chromosomal strands in central region; centriole double. Gilson-Carnoy, haematoxylin.
- FIG. 26. Middle kinetophase. Peripheral view of nucleus shown in Fig. 25. Girdle of endosomal spherules. Gilson-Carnoy, haematoxylin.
- FIG. 27. Middle kinetophase. Chromosomes at poles; endosomal material forming a basophilic reticulum. Gilson-Carnoy, haematoxylin.
- FIG. 28. Middle kinetophase. Chromosomes at poles; endosomal spherules discrete, not forming a reticulum. Flemming, safranin.
- FIG. 29. Late kinetophase. Beginning of nuclear constriction. Gilson-Carnoy, haematoxylin.
- FIG. 30. Late kinetophase. Slightly later than Fig. 29. Gilson-Carnoy, haematoxylin.
- FIG. 31. Late kinetophase. Daughter nucleus after constriction. Gilson-Carnoy, haematoxylin.
- FIG. 32. Late kinetophase. Daughter nucleus beginning to develop toward the interphase. Beginning of the peripheral migration.
- FIG. 33. Nucleus immediately after fixation with Flemming's fixative.
- FIG. 34. Nucleus treated with methyl green.
- FIG. 35. Nucleus immediately after fixation with dioxane.
- FIG. 36. Nucleus immediately after fixation with osmic tetroxide.
- FIG. 37. Living nucleus, early interphase.
- FIG. 38. Same nucleus as Fig. 37, immediately after treatment with absolute alcohol. Note hyaline body at pole.



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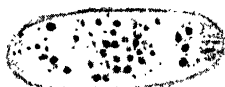
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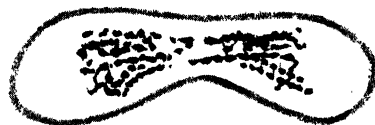
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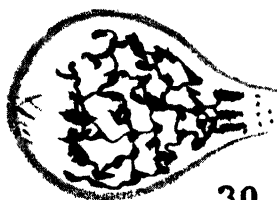
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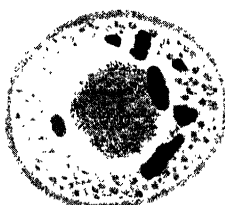
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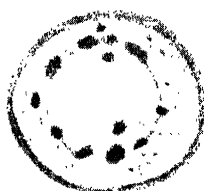
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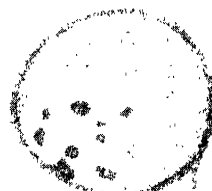
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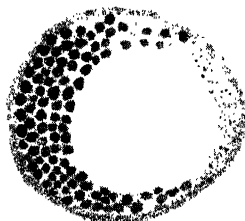
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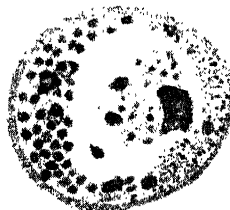
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PLATE III

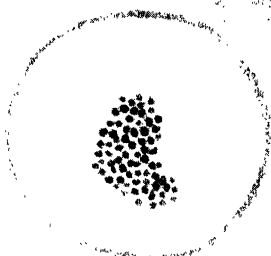
- FIG. 39. Section through whole amoeba. Gilson-Carnoy, haematoxylin.
FIG. 40. Nucleus 7 minutes after being placed in 10 per cent potassium chloride.
FIG. 41. Same nucleus after 12 minutes in 10 per cent potassium chloride.
FIG. 42. Nucleus immediately after fixation with Gilson-Carnoy and staining with methyl green.
FIG. 43. Peripheral region of nucleus fixed in cold absolute alcohol. Unstained peripheral spherules present. Haematoxylin.
FIG. 44. Early kinetophase nucleus fixed in cold absolute alcohol. Chromosomal strands visible in central region. Haematoxylin.
FIG. 45. Middle interphase nucleus fixed in hot absolute alcohol. Endosomes poorly preserved; central region homogeneous. Haematoxylin.
FIG. 46. Peripheral region of nucleus shown in Fig. 45.
FIG. 47. Dividing nucleus fixed in 70 per cent alcohol. Shape irregular due to shrinkage. Haematoxylin.
FIG. 48. Late kinetophase nucleus fixed in hot absolute alcohol. Chromosomes unstained. Feulgen.
FIG. 49. Nucleus fixed in 10 per cent acetic acid. Endosomes and peripheral granules well preserved. Haematoxylin.
FIG. 50. Reorganizing nucleus fixed in 10 per cent acetic acid. Chromosomes well preserved. Haematoxylin.



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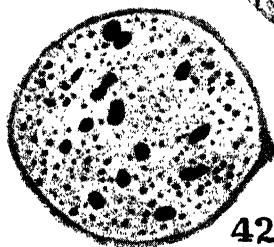
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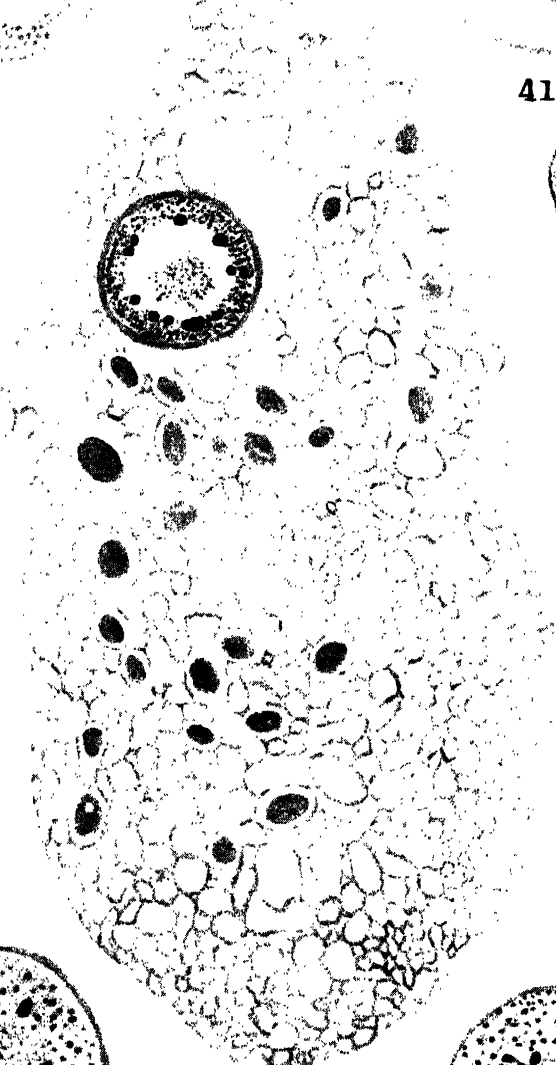
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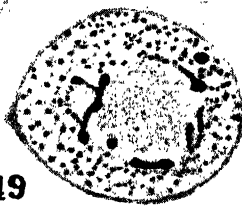
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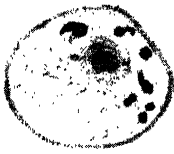
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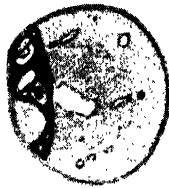
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PLATE IV

- FIG. 51. Nucleus fixed in hot glacial acetic acid. Endosomes incompletely preserved. Haematoxylin.
- FIG. 52. Nucleus fixed in cold glacial acetic acid. Centriole present in homogeneous central region. Haematoxylin.
- FIG. 53. Nucleus fixed in cold glacial acetic acid. Endosomes fused. Haematoxylin.
- FIG. 54. Nucleus fixed in hot formalin. Peripheral spherules and endosomes well preserved. Haematoxylin.
- FIG. 55. Dividing nucleus fixed in hot formalin. Chromosomes indistinguishable as such, but region well stained. Feulgen.
- FIG. 56. Nucleus parasitized by *Nucleophaga* fixed in hot formalin. Haematoxylin.
- FIG. 57. Early kinetophase nucleus fixed in 1 per cent chromic acid. Haematoxylin.
- FIG. 58. Interphase nucleus fixed in 1 per cent chromic acid. No differentiation of peripheral and central ground-plasms. Haematoxylin.
- FIG. 59. Late kinetophase, trophic nucleus. Gilson-Carnoy, haematoxylin.
- FIG. 60. Late kinetophase, trophic nucleus. Gilson-Carnoy, haematoxylin.
- FIG. 61. Late kinetophase, trophic nucleus approaching the precystic condition. Gilson-Carnoy, haematoxylin.
- FIG. 62. Late kinetophase, trophic nucleus nearing precystic condition. Note chromosomes forming reticulum and segregation of peripheral and central materials. Gilson-Carnoy, haematoxylin.
- FIG. 63. Late kinetophase nucleus just before the precystic condition is reached. Very small amount of peripheral material. Gilson-Carnoy, haematoxylin.
- FIG. 64. Late kinetophase, precystic amoeba, binucleate. Gilson-Carnoy, haematoxylin.
- FIG. 65. Interphase, binucleate precystic amoeba. Endosomal material scarce. Small amount of food in cytoplasm. Gilson-Carnoy, haematoxylin.
- FIG. 66. Trinucleate precystic amoeba. Nuclei preparing to divide; almost no food in cytoplasm. Gilson-Carnoy, haematoxylin.
- FIG. 67. Dividing precystic nucleus. Mercuric chloride, haematoxylin.
- FIG. 68. Dividing precystic nucleus. Bouin, haematoxylin.



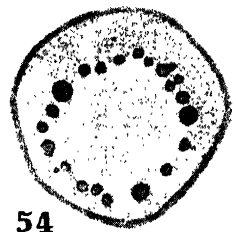
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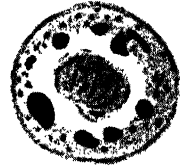
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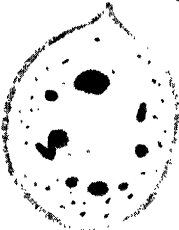
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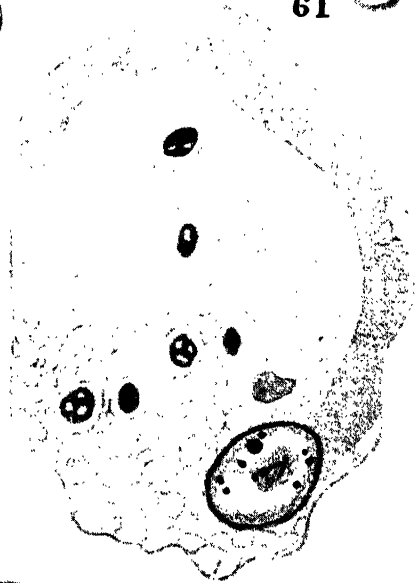
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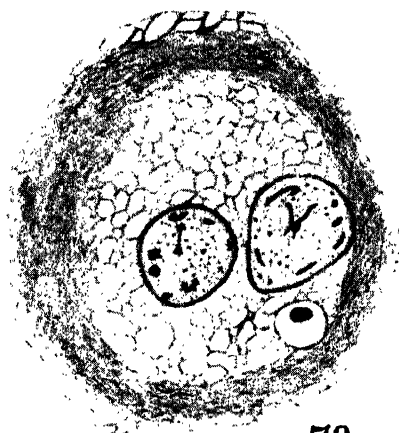
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PLATE V

- FIG. 69. Early precystic divisions, late kinetophase. Gilson-Carnoy, haematoxylin.
- FIG. 70. Precystic interphase, before nuclear transformation. Gilson-Carnoy, haematoxylin.
- FIG. 71. Beginning of nuclear transformation. Appearance of eccentric granule. Gilson-Carnoy, haematoxylin.
- FIG. 72. Nuclear transformation. Enlargement of the eccentric granule. Flemming, safranin.
- FIG. 73. Nuclear reconstruction before transformation. Flemming, safranin.



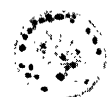
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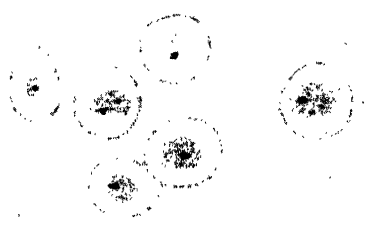


PLATE VI

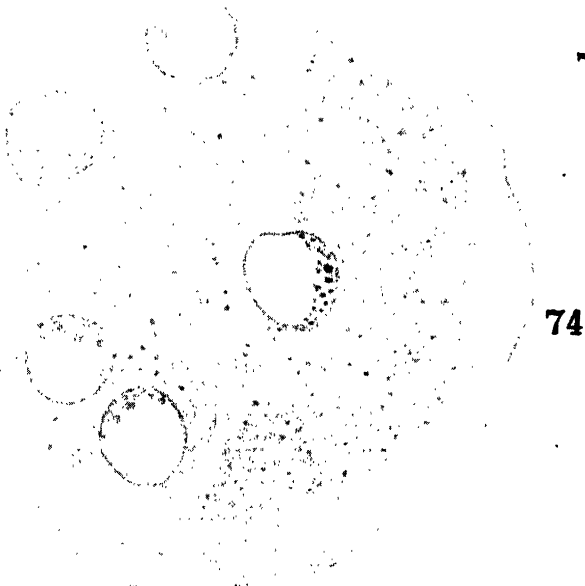
- FIG. 74. Precystic amoeba stained with neutral red. Chromophilic granules present, but none of the larger spherical inclusions. Food vacuoles lacking.
- FIG. 75. Nuclear transformation. Formation of the circlet. Flemming, safranin.
- FIG. 76. Precystic amoeba after completion of nuclear transformation. Gilson-Carnoy, haematoxylin.
- FIG. 77. Division of the circlet. Gilson-Carnoy, haematoxylin.
- FIG. 78. Division of the circlet. Zenker, haematoxylin.



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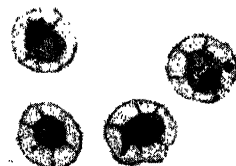
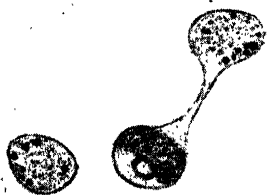


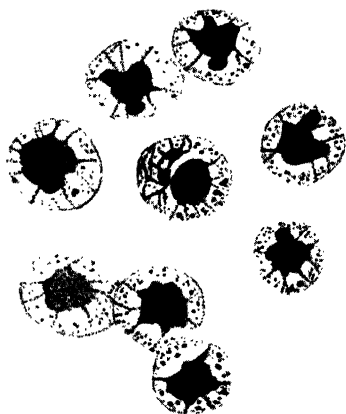
PLATE VII

- FIG. 79. Circlet migrating to poles of central region. Gilson-Carnoy, haematoxylin.
FIG. 80. Nuclei after division in late precystic period. Gilson-Carnoy, haematoxylin.
FIG. 81. Late precystic division figure. No chromosomes, circlet at pole. Gilson-Carnoy, haematoxylin.
FIG. 82. Nucleus at time of cyst wall formation.
FIG. 83. Nuclear appearance at time of cyst wall formation. Flemming, safranin.

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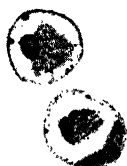
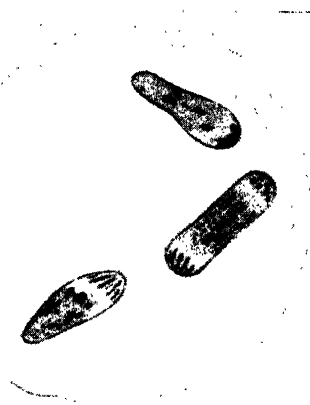


PLATE VIII

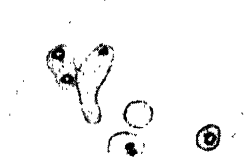
- FIG. 84. Nuclear division immediately after cyst wall formation. Gilson-Carnoy, Feulgen.
- FIG. 85. Cystic division resembling trophic division. Gilson-Carnoy, haematoxylin.
- FIG. 86. Cystic division resembling late precystic division. Bouin, haematoxylin.
- FIG. 87. Next section of same cyst. Bouin, haematoxylin.
- FIG. 88. Cyst with different-sized nuclei. Mercuric chloride, haematoxylin.
- FIG. 89. Mature cyst nuclei. Amitotic (?) division of nuclei. Gilson-Carnoy, haematoxylin.
- FIG. 90. Mature cyst. Hot glacial acetic acid, haematoxylin.
- FIG. 91. Mature cyst. Gilson-Carnoy, haematoxylin.



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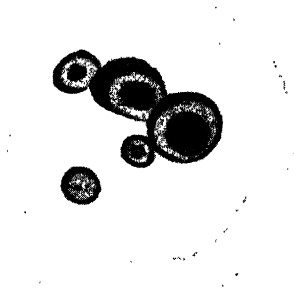
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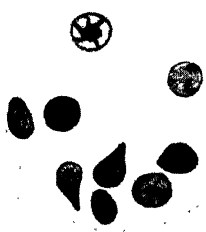
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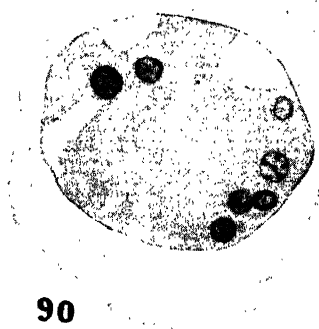
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